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(54) Title: RNA INTERFERENCE MEDIATED INHIBITION OF HYPOXIA INDUCIBLE FACTOR 1 (HIF1) GENE EXPRESSION USING SHORT INTERFERING NUCLEIC ACID (siNA)

(57) Abstract: This invention relates to compounds, compositions, and methods useful for modulating hypoxia inducible factor (e.g., HIF1) gene expression using short interfering nucleic acid (siNA) molecules. This invention also relates to compounds, compositions, and methods useful for modulating the expression and activity of other genes involved in pathways of HIF1 gene expression and/or activity by RNA interference (RNAi) using small nucleic acid molecules. In particular, the instant invention features small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules and methods used to modulate the expression of HIF1 genes.

WO 2005/035759 A2

**RNA INTERFERENCE MEDIATED INHIBITION OF HYPOXIA INDUCIBLE
FACTOR 1 (HIF1) GENE EXPRESSION USING SHORT INTERFERING
NUCLEIC ACID (siNA)**

This application claims the benefit of U.S. Provisional Application No. 5 60/496,655, filed August 20, 2003. This application is a continuation-in-part of International Patent Application No. PCT/US04/16390, filed May 24, 2004, which is a continuation-in-part of U.S. Patent Application No. 10/826,966, filed April 16, 2004, which is continuation-in-part of U.S. Patent Application No. 10/757,803, filed January 14, 2004, which is a continuation-in-part of U.S. Patent Application No. 10/720,448, 10 filed November 24, 2003, which is a continuation-in-part of U.S. Patent Application No. 10/693,059, filed October 23, 2003, which is a continuation-in-part of U.S. Patent Application No. 10/444,853, filed May 23, 2003, which is a continuation-in-part of International Patent Application No. PCT/US03/05346, filed February 20, 2003, and a continuation-in-part of International Patent Application No. PCT/US03/05028, filed 15 February 20, 2003, both of which claim the benefit of U.S. Provisional Application No. 60/358,580 filed February 20, 2002, U.S. Provisional Application No. 60/363,124 filed March 11, 2002, U.S. Provisional Application No. 60/386,782 filed June 6, 2002, U.S. Provisional Application No. 60/406,784 filed August 29, 2002, U.S. Provisional Application No. 60/408,378 filed September 5, 2002, U.S. Provisional Application No. 20 60/409,293 filed September 9, 2002, and U.S. Provisional Application No. 60/440,129 filed January 15, 2003. This application is also a continuation-in-part of International Patent Application No. PCT/US04/13456, filed April 30, 2004, which is a continuation-in-part of U.S. Patent Application No. 10/780,447, filed February 13, 2004, which is a continuation-in-part of U.S. Patent Application No. 10/427,160, filed April 30, 2003, 25 which is a continuation-in-part of International Patent Application No. PCT/US02/15876 filed May 17, 2002, which claims the benefit of U.S. Provisional Application No. 60/292,217, filed May 18, 2001, U.S. Provisional Application No. 60/362,016, filed March 6, 2002, U.S. Provisional Application No. 60/306,883, filed July 20, 2001, and U.S. Provisional Application No. 60/311,865, filed August 13, 2001. This application is 30 also a continuation-in-part of U.S. Patent Application No. 10/727,780 filed December 3, 2003. This application also claims the benefit of U.S. Provisional Application No. 60/543,480, filed February 10, 2004.

The instant application claims the benefit of all the listed applications, which are hereby incorporated by reference herein in their entireties, including the drawings.

Field Of The Invention

The present invention relates to compounds, compositions, and methods for the study, diagnosis, and treatment of traits, diseases and conditions that respond to the modulation of hypoxia-inducible factor-1 (HIF1) gene expression and/or activity. The present invention is also directed to compounds, compositions, and methods relating to traits, diseases and conditions that respond to the modulation of expression and/or activity of genes involved in HIF1 gene expression pathways or other cellular processes that mediate the maintenance or development of such traits, diseases and conditions. Specifically, the invention relates to small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of mediating RNA interference (RNAi) against HIF1 gene expression. Such small nucleic acid molecules are useful, for example, in providing compositions for treatment of traits, diseases and conditions that can respond to modulation of HIF1 expression in a subject, such as cancer, including blood vessel tumors (haemangioblastomas); tumors in the adrenal glands; clear-cell kidney cancers; von Hippel-Lindau (VHL) disease; ovarian cancer; cancers of non-lymphoid parenchymal organs, including the heart, placenta, skeletal muscle and lung; breast cancer; cancers of the head and neck, including various lymphomas such as mantle cell lymphoma, non-Hodgkins lymphoma, adenoma, squamous cell carcinoma, laryngeal carcinoma; cancers of the retina; cancers of the esophagus; multiple myeloma; melanoma; colorectal cancer; lung cancer; bladder cancer; prostate cancer; glioblastoma, proliferative diseases and conditions such as macular degeneration, diabetic retinopathy or any other disease or condition associated with hypoxia inducible proliferation.

Background Of The Invention

The following is a discussion of relevant art pertaining to RNAi. The discussion is provided only for understanding of the invention that follows. The summary is not an admission that any of the work described below is prior art to the claimed invention.

RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Zamore *et al.*, 2000, *Cell*, 101, 25-33; Fire *et al.*, 1998, *Nature*, 391, 806; Hamilton *et al.*, 1999, *Science*, 286, 950-951; Lin *et al.*, 1999, *Nature*, 402, 128-129; Sharp, 1999, *Genes & Dev.*, 13:139-141; and Strauss, 1999, *Science*, 286, 886). The corresponding process in plants (Heifetz *et al.*, International PCT Publication No. WO 99/61631) is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes and is commonly shared by diverse flora and phyla (Fire *et al.*, 1999, *Trends Genet.*, 15, 358). Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or from the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response through a mechanism that has yet to be fully characterized. This mechanism appears to be different from other known mechanisms involving double stranded RNA-specific ribonucleases, such as the interferon response that results from dsRNA-mediated activation of protein kinase PKR and 2',5'-oligoadenylate synthetase resulting in non-specific cleavage of mRNA by ribonuclease L (see for example US Patent Nos. 6,107,094; 5,898,031; Clemens *et al.*, 1997, *J. Interferon & Cytokine Res.*, 17, 503-524; Adah *et al.*, 2001, *Curr. Med. Chem.*, 8, 1189).

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer (Bass, 2000, *Cell*, 101, 235; Zamore *et al.*, 2000, *Cell*, 101, 25-33; Hammond *et al.*, 2000, *Nature*, 404, 293). Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs) (Zamore *et al.*, 2000, *Cell*, 101, 25-33; Bass, 2000, *Cell*, 101, 235; Bernstein *et al.*, 2001, *Nature*, 409, 363). Short interfering RNAs derived from dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes (Zamore *et al.*, 2000, *Cell*, 101, 25-33; Elbashir *et al.*, 2001, *Genes Dev.*, 15, 188). Dicer has also been implicated in the excision of 21- and 22-nucleotide small temporal RNAs (stRNAs) from precursor RNA of conserved structure that are implicated in translational control (Hutvagner *et al.*, 2001, *Science*, 293, 834). The RNAi response also features an endonuclease complex, commonly referred to as an RNA-induced silencing complex

(RISC), which mediates cleavage of single-stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex (Elbashir *et al.*, 2001, *Genes Dev.*, 15, 188).

5 RNAi has been studied in a variety of systems. Fire *et al.*, 1998, *Nature*, 391, 806, were the first to observe RNAi in *C. elegans*. Bahramian and Zarbl, 1999, *Molecular and Cellular Biology*, 19, 274-283 and Wianny and Goetz, 1999, *Nature Cell Biol.*, 2, 70, describe RNAi mediated by dsRNA in mammalian systems. Hammond *et al.*, 2000, *Nature*, 404, 293, describe RNAi in *Drosophila* cells transfected with dsRNA. Elbashir
10 *et al.*, 2001, *Nature*, 411, 494 and Tuschl *et al.*, International PCT Publication No. WO 01/75164, describe RNAi induced by introduction of duplexes of synthetic 21-nucleotide RNAs in cultured mammalian cells including human embryonic kidney and HeLa cells. Recent work in *Drosophila* embryonic lysates (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877 and Tuschl *et al.*, International PCT Publication No. WO 01/75164) has revealed certain
15 requirements for siRNA length, structure, chemical composition, and sequence that are essential to mediate efficient RNAi activity. These studies have shown that 21-nucleotide siRNA duplexes are most active when containing 3'-terminal dinucleotide overhangs. Furthermore, complete substitution of one or both siRNA strands with 2'-deoxy (2'-H) or 2'-O-methyl nucleotides abolishes RNAi activity, whereas substitution of
20 the 3'-terminal siRNA overhang nucleotides with 2'-deoxy nucleotides (2'-H) was shown to be tolerated. Single mismatch sequences in the center of the siRNA duplex were also shown to abolish RNAi activity. In addition, these studies also indicate that the position of the cleavage site in the target RNA is defined by the 5'-end of the siRNA guide sequence rather than the 3'-end of the guide sequence (Elbashir *et al.*, 2001, *EMBO J.*,
25 20, 6877). Other studies have indicated that a 5'-phosphate on the target-complementary strand of a siRNA duplex is required for siRNA activity and that ATP is utilized to maintain the 5'-phosphate moiety on the siRNA (Nykanen *et al.*, 2001, *Cell*, 107, 309).

Studies have shown that replacing the 3'-terminal nucleotide overhanging segments of a 21-mer siRNA duplex having two-nucleotide 3'-overhangs with
30 deoxyribonucleotides does not have an adverse effect on RNAi activity. Replacing up to four nucleotides on each end of the siRNA with deoxyribonucleotides has been reported to be well tolerated, whereas complete substitution with deoxyribonucleotides results in no RNAi activity (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877 and Tuschl *et al.*,

International PCT Publication No. WO 01/75164). In addition, Elbashir *et al.*, *supra*, also report that substitution of siRNA with 2'-O-methyl nucleotides completely abolishes RNAi activity. Li *et al.*, International PCT Publication No. WO 00/44914, and Beach *et al.*, International PCT Publication No. WO 01/68836 preliminarily suggest that siRNA
5 may include modifications to either the phosphate-sugar backbone or the nucleoside to include at least one of a nitrogen or sulfur heteroatom, however, neither application postulates to what extent such modifications would be tolerated in siRNA molecules, nor provides any further guidance or examples of such modified siRNA. Kreutzer *et al.*, Canadian Patent Application No. 2,359,180, also describe certain chemical modifications
10 for use in dsRNA constructs in order to counteract activation of double-stranded RNA-dependent protein kinase PKR, specifically 2'-amino or 2'-O-methyl nucleotides, and nucleotides containing a 2'-O or 4'-C methylene bridge. However, Kreutzer *et al.* similarly fails to provide examples or guidance as to what extent these modifications would be tolerated in dsRNA molecules.

15 Parrish *et al.*, 2000, *Molecular Cell*, 6, 1077-1087, tested certain chemical modifications targeting the unc-22 gene in *C. elegans* using long (>25 nt) siRNA transcripts. The authors describe the introduction of thiophosphate residues into these siRNA transcripts by incorporating thiophosphate nucleotide analogs with T7 and T3 RNA polymerase and observed that RNAs with two phosphorothioate modified bases
20 also had substantial decreases in effectiveness as RNAi. Further, Parrish *et al.* reported that phosphorothioate modification of more than two residues greatly destabilized the RNAs *in vitro* such that interference activities could not be assayed. *Id.* at 1081. The authors also tested certain modifications at the 2'-position of the nucleotide sugar in the long siRNA transcripts and found that substituting deoxynucleotides for ribonucleotides
25 produced a substantial decrease in interference activity, especially in the case of Uridine to Thymidine and/or Cytidine to deoxy-Cytidine substitutions. *Id.* In addition, the authors tested certain base modifications, including substituting, in sense and antisense strands of the siRNA, 4-thiouracil, 5-bromouracil, 5-iodouracil, and 3-(aminoallyl)uracil for uracil, and inosine for guanosine. Whereas 4-thiouracil and 5-bromouracil
30 substitution appeared to be tolerated, Parrish reported that inosine produced a substantial decrease in interference activity when incorporated in either strand. Parrish also reported that incorporation of 5-iodouracil and 3-(aminoallyl)uracil in the antisense strand resulted in a substantial decrease in RNAi activity as well.

The use of longer dsRNA has been described. For example, Beach *et al.*, International PCT Publication No. WO 01/68836, describes specific methods for attenuating gene expression using endogenously-derived dsRNA. Tuschl *et al.*, International PCT Publication No. WO 01/75164, describe a *Drosophila in vitro* RNAi system and the use of specific siRNA molecules for certain functional genomic and certain therapeutic applications; although Tuschl, 2001, *Chem. Biochem.*, 2, 239-245, doubts that RNAi can be used to cure genetic diseases or viral infection due to the danger of activating interferon response. Li *et al.*, International PCT Publication No. WO 00/44914, describe the use of specific long (141 bp-488 bp) enzymatically synthesized or vector expressed dsRNAs for attenuating the expression of certain target genes. Zernicka-Goetz *et al.*, International PCT Publication No. WO 01/36646, describe certain methods for inhibiting the expression of particular genes in mammalian cells using certain long (550 bp-714 bp), enzymatically synthesized or vector expressed dsRNA molecules. Fire *et al.*, International PCT Publication No. WO 99/32619, describe particular methods for introducing certain long dsRNA molecules into cells for use in inhibiting gene expression in nematodes. Plaetinck *et al.*, International PCT Publication No. WO 00/01846, describe certain methods for identifying specific genes responsible for conferring a particular phenotype in a cell using specific long dsRNA molecules. Mello *et al.*, International PCT Publication No. WO 01/29058, describe the identification of specific genes involved in dsRNA-mediated RNAi. Pachuck *et al.*, International PCT Publication No. WO 00/63364, describe certain long (at least 200 nucleotide) dsRNA constructs. Deschamps Depaillette *et al.*, International PCT Publication No. WO 99/07409, describe specific compositions consisting of particular dsRNA molecules combined with certain anti-viral agents. Waterhouse *et al.*, International PCT Publication No. 99/53050 and 1998, *PNAS*, 95, 13959-13964, describe certain methods for decreasing the phenotypic expression of a nucleic acid in plant cells using certain dsRNAs. Driscoll *et al.*, International PCT Publication No. WO 01/49844, describe specific DNA expression constructs for use in facilitating gene silencing in targeted organisms.

Others have reported on various RNAi and gene-silencing systems. For example, Parrish *et al.*, 2000, *Molecular Cell*, 6, 1077-1087, describe specific chemically-modified dsRNA constructs targeting the *unc-22* gene of *C. elegans*. Grossniklaus, International PCT Publication No. WO 01/38551, describes certain methods for regulating polycomb gene expression in plants using certain dsRNAs. Churikov *et al.*, International PCT

Publication No. WO 01/42443, describe certain methods for modifying genetic characteristics of an organism using certain dsRNAs. Cogoni *et al.*, International PCT Publication No. WO 01/53475, describe certain methods for isolating a *Neurospora* silencing gene and uses thereof. Reed *et al.*, International PCT Publication No. WO 01/68836, describe certain methods for gene silencing in plants. Honer *et al.*, International PCT Publication No. WO 01/70944, describe certain methods of drug screening using transgenic nematodes as Parkinson's Disease models using certain dsRNAs. Deak *et al.*, International PCT Publication No. WO 01/72774, describe certain *Drosophila*-derived gene products that may be related to RNAi in *Drosophila*. Arndt *et al.*, International PCT Publication No. WO 01/92513 describe certain methods for mediating gene suppression by using factors that enhance RNAi. Tuschl *et al.*, International PCT Publication No. WO 02/44321, describe certain synthetic siRNA constructs. Pachuk *et al.*, International PCT Publication No. WO 00/63364, and Satishchandran *et al.*, International PCT Publication No. WO 01/04313, describe certain methods and compositions for inhibiting the function of certain polynucleotide sequences using certain long (over 250 bp), vector expressed dsRNAs. Echeverri *et al.*, International PCT Publication No. WO 02/38805, describe certain *C. elegans* genes identified via RNAi. Kreutzer *et al.*, International PCT Publications Nos. WO 02/055692, WO 02/055693, and EP 1144623 B1 describes certain methods for inhibiting gene expression using dsRNA. Graham *et al.*, International PCT Publications Nos. WO 99/49029 and WO 01/70949, and AU 4037501 describe certain vector expressed siRNA molecules. Fire *et al.*, US 6,506,559, describe certain methods for inhibiting gene expression in vitro using certain long dsRNA (299 bp-1033 bp) constructs that mediate RNAi. Martinez *et al.*, 2002, *Cell*, 110, 563-574, describe certain single stranded siRNA constructs, including certain 5'-phosphorylated single stranded siRNAs that mediate RNA interference in HeLa cells. Harborth *et al.*, 2003, *Antisense & Nucleic Acid Drug Development*, 13, 83-105, describe certain chemically and structurally modified siRNA molecules. Chiu and Rana, 2003, *RNA*, 9, 1034-1048, describe certain chemically and structurally modified siRNA molecules. Woolf *et al.*, International PCT Publication Nos. WO 03/064626 and WO 03/064625 describe certain chemically modified dsRNA constructs.

SUMMARY OF THE INVENTION

This invention relates to compounds, compositions, and methods useful for modulating hypoxia-inducible factor genes, such as hypoxia-inducible factor-1 (HIF1, e.g., HIF1-alpha and/or HIF1-beta) gene expression using short interfering nucleic acid (siNA) molecules. This invention also relates to compounds, compositions, and methods useful for modulating the expression and activity of other genes involved in pathways of HIF1 gene expression and/or activity by RNA interference (RNAi) using small nucleic acid molecules. In particular, the instant invention features small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules and methods used to modulate the expression of HIF1 genes.

A siNA of the invention can be unmodified or chemically-modified. A siNA of the instant invention can be chemically synthesized, expressed from a vector or enzymatically synthesized. The instant invention also features various chemically-modified synthetic short interfering nucleic acid (siNA) molecules capable of modulating HIF1 gene expression or activity in cells by RNA interference (RNAi). The use of chemically-modified siNA improves various properties of native siNA molecules through increased resistance to nuclease degradation *in vivo* and/or through improved cellular uptake. Further, contrary to earlier published studies, siNA having multiple chemical modifications retains its RNAi activity. The siNA molecules of the instant invention provide useful reagents and methods for a variety of therapeutic, diagnostic, target validation, genomic discovery, genetic engineering, and pharmacogenomic applications.

In one embodiment, the invention features one or more siNA molecules and methods that independently or in combination modulate the expression of HIF1 genes encoding proteins, such as proteins comprising HIF1 associated with the maintenance and/or development of cancer and/or proliferative diseases and conditions, such as genes encoding sequences comprising those sequences referred to by GenBank Accession Nos. shown in Table I, (e.g., HIF1-alpha, HIF1-beta, HIF2, and/or HIF3) referred to herein generally as HIF1. The description below of the various aspects and embodiments of the invention is provided with reference to exemplary HIF1 genes referred to herein as HIF1-alpha, HIF1-beta, or HIF1. However, the various aspects and embodiments are

also directed to other HIF1 genes, such as HIF1 homolog genes and transcript variants including HIF2 and/or HIF3, and polymorphisms (e.g., single nucleotide polymorphism, (SNPs)) associated with certain HIF1 genes. As such, the various aspects and embodiments are also directed to other genes that are involved in HIF1 mediated pathways of signal transduction or gene expression that, for example, encode any HIF1 ligands and receptors. These additional genes can be analyzed for target sites using the methods described for HIF1 genes herein. Thus, the modulation of other genes and the effects of such modulation of the other genes can be performed, determined, and measured as described herein.

10 In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a HIF1 gene, wherein said siNA molecule comprises about 15 to about 28 base pairs.

In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that directs cleavage of a HIF1 RNA via RNA interference (RNAi), wherein the double stranded siNA molecule comprises a first and a second strand, each strand of the siNA molecule is about 18 to about 28 nucleotides in length, the first strand of the siNA molecule comprises nucleotide sequence having sufficient complementarity to the HIF1 RNA for the siNA molecule to direct cleavage of the HIF1 RNA via RNA interference, and the second strand of said siNA molecule comprises nucleotide sequence that is complementary to the first strand.

In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that directs cleavage of a HIF1 RNA via RNA interference (RNAi), wherein the double stranded siNA molecule comprises a first and a second strand, each strand of the siNA molecule is about 18 to about 23 nucleotides in length, the first strand of the siNA molecule comprises nucleotide sequence having sufficient complementarity to the HIF1 RNA for the siNA molecule to direct cleavage of the HIF1 RNA via RNA interference, and the second strand of said siNA molecule comprises nucleotide sequence that is complementary to the first strand.

In one embodiment, the invention features a chemically synthesized double stranded short interfering nucleic acid (siNA) molecule that directs cleavage of a HIF1 RNA via RNA interference (RNAi), wherein each strand of the siNA molecule is about 18 to about 28 nucleotides in length; and one strand of the siNA molecule comprises

nucleotide sequence having sufficient complementarity to the HIF1 RNA for the siNA molecule to direct cleavage of the HIF1 RNA via RNA interference.

In one embodiment, the invention features a chemically synthesized double stranded short interfering nucleic acid (siNA) molecule that directs cleavage of a HIF1 RNA via RNA interference (RNAi), wherein each strand of the siNA molecule is about 18 to about 23 nucleotides in length; and one strand of the siNA molecule comprises nucleotide sequence having sufficient complementarity to the HIF1 RNA for the siNA molecule to direct cleavage of the HIF1 RNA via RNA interference.

In one embodiment, the invention features a siNA molecule that down-regulates expression of a HIF1 gene, for example, wherein the HIF1 gene comprises HIF1 encoding sequence. In one embodiment, the invention features a siNA molecule that down-regulates expression of a HIF1 gene, for example, wherein the HIF1 gene comprises HIF1 non-coding sequence or regulatory elements involved in HIF1 gene expression.

In one embodiment, a siNA of the invention is used to inhibit the expression of HIF1 genes or a HIF1 gene family, wherein the genes or gene family sequences share sequence homology. Such homologous sequences can be identified as is known in the art, for example using sequence alignments. siNA molecules can be designed to target such homologous sequences, for example using perfectly complementary sequences or by incorporating non-canonical base pairs, for example mismatches and/or wobble base pairs, that can provide additional target sequences. In instances where mismatches are identified, non-canonical base pairs (for example, mismatches and/or wobble bases) can be used to generate siNA molecules that target more than one gene sequence. In a non-limiting example, non-canonical base pairs such as UU and CC base pairs are used to generate siNA molecules that are capable of targeting sequences for differing HIF1 targets that share sequence homology (e.g., HIF2, HIF3). As such, one advantage of using siNAs of the invention is that a single siNA can be designed to include nucleic acid sequence that is complementary to the nucleotide sequence that is conserved between the homologous genes. In this approach, a single siNA can be used to inhibit expression of more than one gene instead of using more than one siNA molecule to target the different genes.

In one embodiment, the invention features a siNA molecule having RNAi activity against HIF1 RNA, wherein the siNA molecule comprises a sequence complementary to any RNA having HIF1 encoding sequence, such as those sequences having GenBank Accession Nos. shown in Table I. In another embodiment, the invention features a siNA molecule having RNAi activity against HIF1 RNA, wherein the siNA molecule comprises a sequence complementary to an RNA having variant HIF1 encoding sequence, for example other mutant HIF1 genes not shown in Table I but known in the art to be associated with the maintenance and/or development of cancer and/or proliferative diseases and conditions. Chemical modifications as shown in Tables III and IV or otherwise described herein can be applied to any siNA construct of the invention. In another embodiment, a siNA molecule of the invention includes a nucleotide sequence that can interact with nucleotide sequence of a HIF1 gene and thereby mediate silencing of HIF1 gene expression, for example, wherein the siNA mediates regulation of HIF1 gene expression by cellular processes that modulate the chromatin structure or methylation patterns of the HIF1 gene and prevent transcription of the HIF1 gene.

In one embodiment, siNA molecules of the invention are used to down regulate or inhibit the expression of HIF1 proteins arising from HIF1 haplotype polymorphisms that are associated with a disease or condition, (e.g., cancer and/or proliferative diseases and conditions). Analysis of HIF1 genes, or HIF1 protein or RNA levels can be used to identify subjects with such polymorphisms or those subjects who are at risk of developing traits, conditions, or diseases described herein. These subjects are amenable to treatment, for example, treatment with siNA molecules of the invention and any other composition useful in treating diseases related to HIF1 gene expression. As such, analysis of HIF1 protein or RNA levels can be used to determine treatment type and the course of therapy in treating a subject. Monitoring of HIF1 protein or RNA levels can be used to predict treatment outcome and to determine the efficacy of compounds and compositions that modulate the level and/or activity of certain HIF1 proteins associated with a trait, condition, or disease.

In one embodiment of the invention a siNA molecule comprises an antisense strand comprising a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof encoding a HIF1 protein. The siNA further comprises a sense strand,

wherein said sense strand comprises a nucleotide sequence of a HIF1 gene or a portion thereof.

5 In another embodiment, a siNA molecule comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence encoding a HIF1 protein or a portion thereof. The siNA molecule further comprises a sense region, wherein said sense region comprises a nucleotide sequence of a HIF1 gene or a portion thereof.

10 In another embodiment, the invention features a siNA molecule comprising a nucleotide sequence in the antisense region of the siNA molecule that is complementary to a nucleotide sequence or portion of sequence of a HIF1 gene. In another embodiment, the invention features a siNA molecule comprising a region, for example, the antisense region of the siNA construct, complementary to a sequence comprising a HIF1 gene sequence or a portion thereof.

15 In one embodiment, the antisense region of HIF1 siNA constructs comprises a sequence complementary to sequence having any of SEQ ID NOs. 1-220, 441-456, 465-472, 481-488, 497-504, 513-520, 545, 547, 549, 551, 552, 554, 556, 558, 560, or 561. In one embodiment, the antisense region of HIF1 constructs comprises sequence having any of SEQ ID NOs. 221-440, 457-464, 473-480, 489-496, 505-512, 521-544, 546, 548, 550, 553, 555, 557, 559, or 562. In another embodiment, the sense region of HIF1 constructs
20 comprises sequence having any of SEQ ID NOs. 1-220, 441-456, 465-472, 481-488, 497-504, 513-520, 545, 547, 549, 551, 552, 554, 556, 558, 560, or 561.

In one embodiment, a siNA molecule of the invention comprises any of SEQ ID NOs. 1-562. The sequences shown in SEQ ID NOs: 1-562 are not limiting. A siNA molecule of the invention can comprise any contiguous HIF1 sequence (e.g., about 15 to
25 about 25 or more, or about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 or more contiguous HIF1 nucleotides).

In yet another embodiment, the invention features a siNA molecule comprising a sequence, for example, the antisense sequence of the siNA construct, complementary to a sequence or portion of sequence comprising sequence represented by GenBank
30 Accession Nos. shown in Table I. Chemical modifications in Tables III and IV and described herein can be applied to any siNA construct of the invention.

In one embodiment of the invention a siNA molecule comprises an antisense strand having about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, wherein the antisense strand is complementary to a RNA sequence or a portion thereof encoding a HIF1 protein, and wherein said siNA
5 further comprises a sense strand having about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, and wherein said sense strand and said antisense strand are distinct nucleotide sequences where at least about 15 nucleotides in each strand are complementary to the other strand.

In another embodiment of the invention a siNA molecule of the invention
10 comprises an antisense region having about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, wherein the antisense region is complementary to a RNA sequence encoding a HIF1 protein, and wherein said siNA further comprises a sense region having about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, wherein said sense region
15 and said antisense region are comprised in a linear molecule where the sense region comprises at least about 15 nucleotides that are complementary to the antisense region.

In one embodiment, a siNA molecule of the invention has RNAi activity that modulates expression of RNA encoded by a HIF1 gene. Because HIF1 genes can share some degree of sequence homology with each other, siNA molecules can be designed to
20 target a class of HIF1 genes or alternately specific HIF1 genes (e.g., polymorphic variants) by selecting sequences that are either shared amongst different HIF1 targets or alternatively that are unique for a specific HIF1 target. Therefore, in one embodiment, the siNA molecule can be designed to target conserved regions of HIF1 RNA sequences having homology among several HIF1 gene variants so as to target a class of HIF1 genes
25 with one siNA molecule. Accordingly, in one embodiment, the siNA molecule of the invention modulates the expression of one or both HIF1 alleles in a subject. In another embodiment, the siNA molecule can be designed to target a sequence that is unique to a specific HIF1 RNA sequence (e.g., a single HIF1 allele or HIF1 single nucleotide polymorphism (SNP)) due to the high degree of specificity that the siNA molecule
30 requires to mediate RNAi activity.

In one embodiment, nucleic acid molecules of the invention that act as mediators of the RNA interference gene silencing response are double-stranded nucleic acid

molecules. In another embodiment, the siNA molecules of the invention consist of duplex nucleic acid molecules containing about 15 to about 30 base pairs between oligonucleotides comprising about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides. In yet another embodiment, siNA molecules of the invention comprise duplex nucleic acid molecules with overhanging ends of about 1 to about 3 (e.g., about 1, 2, or 3) nucleotides, for example, about 21-nucleotide duplexes with about 19 base pairs and 3'-terminal mononucleotide, dinucleotide, or trinucleotide overhangs. In yet another embodiment, siNA molecules of the invention comprise duplex nucleic acid molecules with blunt ends, where both ends are blunt, or alternatively, where one of the ends is blunt.

In one embodiment, the invention features one or more chemically-modified siNA constructs having specificity for HIF1 expressing nucleic acid molecules, such as RNA encoding a HIF1 protein. In one embodiment, the invention features a RNA based siNA molecule (e.g., a siNA comprising 2'-OH nucleotides) having specificity for HIF1 expressing nucleic acid molecules that includes one or more chemical modifications described herein. Non-limiting examples of such chemical modifications include without limitation phosphorothioate internucleotide linkages, 2'-deoxyribonucleotides, 2'-O-methyl ribonucleotides, 2'-deoxy-2'-fluoro ribonucleotides, "universal base" nucleotides, "acyclic" nucleotides, 5-C-methyl nucleotides, and terminal glyceryl and/or inverted deoxy abasic residue incorporation. These chemical modifications, when used in various siNA constructs, (e.g., RNA based siNA constructs), are shown to preserve RNAi activity in cells while at the same time, dramatically increasing the serum stability of these compounds. Furthermore, contrary to the data published by Parrish *et al.*, *supra*, applicant demonstrates that multiple (greater than one) phosphorothioate substitutions are well-tolerated and confer substantial increases in serum stability for modified siNA constructs.

In one embodiment, a siNA molecule of the invention comprises modified nucleotides while maintaining the ability to mediate RNAi. The modified nucleotides can be used to improve *in vitro* or *in vivo* characteristics such as stability, activity, and/or bioavailability. For example, a siNA molecule of the invention can comprise modified nucleotides as a percentage of the total number of nucleotides present in the siNA molecule. As such, a siNA molecule of the invention can generally comprise about 5% to about 100% modified nucleotides (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, 35%,

40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% modified nucleotides). The actual percentage of modified nucleotides present in a given siNA molecule will depend on the total number of nucleotides present in the siNA. If the siNA molecule is single stranded, the percent modification can be based upon the total number of nucleotides present in the single stranded siNA molecules. Likewise, if the siNA molecule is double stranded, the percent modification can be based upon the total number of nucleotides present in the sense strand, antisense strand, or both the sense and antisense strands.

One aspect of the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a HIF1 gene. In one embodiment, the double stranded siNA molecule comprises one or more chemical modifications and each strand of the double-stranded siNA is about 21 nucleotides long. In one embodiment, the double-stranded siNA molecule does not contain any ribonucleotides. In another embodiment, the double-stranded siNA molecule comprises one or more ribonucleotides. In one embodiment, each strand of the double-stranded siNA molecule independently comprises about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, wherein each strand comprises about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides that are complementary to the nucleotides of the other strand. In one embodiment, one of the strands of the double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of the HIF1 gene, and the second strand of the double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence of the HIF1 gene or a portion thereof.

In another embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a HIF1 gene comprising an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of the HIF1 gene or a portion thereof, and a sense region, wherein the sense region comprises a nucleotide sequence substantially similar to the nucleotide sequence of the HIF1 gene or a portion thereof. In one embodiment, the antisense region and the sense region independently comprise about 15 to about 30 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, wherein the antisense region comprises about 15 to about 30 (e.g. about 15,

16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides that are complementary to nucleotides of the sense region.

In another embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a HIF1 gene comprising
5 a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by the HIF1 gene or a portion thereof and the sense region comprises a nucleotide sequence that is complementary to the antisense region.

In one embodiment, a siNA molecule of the invention comprises blunt ends, i.e.,
10 ends that do not include any overhanging nucleotides. For example, a siNA molecule comprising modifications described herein (e.g., comprising nucleotides having Formulae I-VII or siNA constructs comprising "Stab 00"- "Stab 32" (Table IV) or any combination thereof (see Table IV)) and/or any length described herein can comprise blunt ends or ends with no overhanging nucleotides.

In one embodiment, any siNA molecule of the invention can comprise one or more blunt ends, i.e. where a blunt end does not have any overhanging nucleotides. In one embodiment, the blunt ended siNA molecule has a number of base pairs equal to the number of nucleotides present in each strand of the siNA molecule. In another embodiment, the siNA molecule comprises one blunt end, for example wherein the 5'-
20 end of the antisense strand and the 3'-end of the sense strand do not have any overhanging nucleotides. In another example, the siNA molecule comprises one blunt end, for example wherein the 3'-end of the antisense strand and the 5'-end of the sense strand do not have any overhanging nucleotides. In another example, a siNA molecule comprises two blunt ends, for example wherein the 3'-end of the antisense strand and the
25 5'-end of the sense strand as well as the 5'-end of the antisense strand and 3'-end of the sense strand do not have any overhanging nucleotides. A blunt ended siNA molecule can comprise, for example, from about 15 to about 30 nucleotides (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides). Other nucleotides present in a blunt ended siNA molecule can comprise, for example, mismatches, bulges,
30 loops, or wobble base pairs to modulate the activity of the siNA molecule to mediate RNA interference.

By "blunt ends" is meant symmetric termini or termini of a double stranded siNA molecule having no overhanging nucleotides. The two strands of a double stranded siNA molecule align with each other without over-hanging nucleotides at the termini. For example, a blunt ended siNA construct comprises terminal nucleotides that are
5 complementary between the sense and antisense regions of the siNA molecule.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a HIF1 gene, wherein the siNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the
10 antisense region of the siNA molecule. The sense region can be connected to the antisense region via a linker molecule, such as a polynucleotide linker or a non-nucleotide linker.

In one embodiment, the invention features double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a HIF1 gene, wherein
15 the siNA molecule comprises about 15 to about 30 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) base pairs, and wherein each strand of the siNA molecule comprises one or more chemical modifications. In another embodiment, one of the strands of the double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence of a HIF1 gene or a portion thereof, and the
20 second strand of the double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence or a portion thereof of the HIF1 gene. In another embodiment, one of the strands of the double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence of a HIF1 gene or portion thereof, and the second strand of the double-stranded siNA molecule comprises a
25 nucleotide sequence substantially similar to the nucleotide sequence or portion thereof of the HIF1 gene. In another embodiment, each strand of the siNA molecule comprises about 15 to about 30 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, and each strand comprises at least about 15 to about 30 (e.g. about 15,
30 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides that are complementary to the nucleotides of the other strand. The HIF1 gene can comprise, for example, sequences referred to in Table I.

In one embodiment, a siNA molecule of the invention comprises no ribonucleotides. In another embodiment, a siNA molecule of the invention comprises ribonucleotides.

In one embodiment, a siNA molecule of the invention comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence of a HIF1 gene or a portion thereof, and the siNA further comprises a sense region comprising a nucleotide sequence substantially similar to the nucleotide sequence of the HIF1 gene or a portion thereof. In another embodiment, the antisense region and the sense region each comprise about 15 to about 30 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides and the antisense region comprises at least about 15 to about 30 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides that are complementary to nucleotides of the sense region. The HIF1 gene can comprise, for example, sequences referred to in Table I. In another embodiment, the siNA is a double stranded nucleic acid molecule, where each of the two strands of the siNA molecule independently comprise about 15 to about 40 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40) nucleotides, and where one of the strands of the siNA molecule comprises at least about 15 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 or more) nucleotides that are complementary to the nucleic acid sequence of the HIF1 gene or a portion thereof.

In one embodiment, a siNA molecule of the invention comprises a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by a HIF1 gene, or a portion thereof, and the sense region comprises a nucleotide sequence that is complementary to the antisense region. In one embodiment, the siNA molecule is assembled from two separate oligonucleotide fragments, wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule. In another embodiment, the sense region is connected to the antisense region via a linker molecule. In another embodiment, the sense region is connected to the antisense region via a linker molecule, such as a nucleotide or non-nucleotide linker. The HIF1 gene can comprise, for example, sequences referred in to Table I.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a HIF1 gene comprising a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by the HIF1 gene or a portion thereof and the sense region comprises a nucleotide sequence that is complementary to the antisense region, and wherein the siNA molecule has one or more modified pyrimidine and/or purine nucleotides. In one embodiment, the pyrimidine nucleotides in the sense region are 2'-O-methyl pyrimidine nucleotides or 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-deoxy purine nucleotides. In another embodiment, the pyrimidine nucleotides in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-O-methyl purine nucleotides. In another embodiment, the pyrimidine nucleotides in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-deoxy purine nucleotides. In one embodiment, the pyrimidine nucleotides in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the antisense region are 2'-O-methyl or 2'-deoxy purine nucleotides. In another embodiment of any of the above-described siNA molecules, any nucleotides present in a non-complementary region of the sense strand (e.g. overhang region) are 2'-deoxy nucleotides.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a HIF1 gene, wherein the siNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule, and wherein the fragment comprising the sense region includes a terminal cap moiety at the 5'-end, the 3'-end, or both of the 5' and 3' ends of the fragment. In one embodiment, the terminal cap moiety is an inverted deoxy abasic moiety or glyceryl moiety. In one embodiment, each of the two fragments of the siNA molecule independently comprise about 15 to about 30 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides. In another embodiment, each of the two fragments of the siNA molecule independently comprise about 15 to about 40 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 23, 33, 34, 35, 36, 37, 38, 39, or 40) nucleotides. In a non-limiting example, each of the two fragments of the siNA molecule comprise about 21 nucleotides.

In one embodiment, the invention features a siNA molecule comprising at least one modified nucleotide, wherein the modified nucleotide is a 2'-deoxy-2'-fluoro nucleotide. The siNA can be, for example, about 15 to about 40 nucleotides in length. In one embodiment, all pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides. In one embodiment, the modified nucleotides in the siNA include at least one 2'-deoxy-2'-fluoro cytidine or 2'-deoxy-2'-fluoro uridine nucleotide. In another embodiment, the modified nucleotides in the siNA include at least one 2'-fluoro cytidine and at least one 2'-deoxy-2'-fluoro uridine nucleotides. In one embodiment, all uridine nucleotides present in the siNA are 2'-deoxy-2'-fluoro uridine nucleotides. In one embodiment, all cytidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro cytidine nucleotides. In one embodiment, all adenosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro adenosine nucleotides. In one embodiment, all guanosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro guanosine nucleotides. The siNA can further comprise at least one modified internucleotidic linkage, such as phosphorothioate linkage. In one embodiment, the 2'-deoxy-2'-fluoronucleotides are present at specifically selected locations in the siNA that are sensitive to cleavage by ribonucleases, such as locations having pyrimidine nucleotides.

In one embodiment, the invention features a method of increasing the stability of a siNA molecule against cleavage by ribonucleases comprising introducing at least one modified nucleotide into the siNA molecule, wherein the modified nucleotide is a 2'-deoxy-2'-fluoro nucleotide. In one embodiment, all pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides. In one embodiment, the modified nucleotides in the siNA include at least one 2'-deoxy-2'-fluoro cytidine or 2'-deoxy-2'-fluoro uridine nucleotide. In another embodiment, the modified nucleotides in the siNA include at least one 2'-fluoro cytidine and at least one 2'-deoxy-2'-fluoro uridine nucleotides. In one embodiment, all uridine nucleotides present in the siNA are 2'-deoxy-2'-fluoro uridine nucleotides. In one embodiment, all cytidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro cytidine nucleotides. In one embodiment, all adenosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro adenosine nucleotides. In one embodiment, all guanosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro guanosine nucleotides. The siNA can further comprise at least one modified internucleotidic linkage, such as phosphorothioate linkage. In one embodiment, the 2'-deoxy-2'-fluoronucleotides are present at specifically selected locations in the siNA that

are sensitive to cleavage by ribonucleases, such as locations having pyrimidine nucleotides.

5 In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a HIF1 gene comprising a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by the HIF1 gene or a portion thereof and the sense region comprises a nucleotide sequence that is complementary to the antisense region, and wherein the purine nucleotides present in the antisense region comprise 2'-deoxy- purine nucleotides. In an alternative
10 embodiment, the purine nucleotides present in the antisense region comprise 2'-O-methyl purine nucleotides. In either of the above embodiments, the antisense region can comprise a phosphorothioate internucleotide linkage at the 3' end of the antisense region. Alternatively, in either of the above embodiments, the antisense region can comprise a glyceryl modification at the 3' end of the antisense region. In another embodiment of
15 any of the above-described siNA molecules, any nucleotides present in a non-complementary region of the antisense strand (e.g. overhang region) are 2'-deoxy nucleotides.

In one embodiment, the antisense region of a siNA molecule of the invention comprises sequence complementary to a portion of a HIF1 transcript having sequence
20 unique to a particular HIF1 disease related allele, such as sequence comprising a single nucleotide polymorphism (SNP) associated with the disease specific allele. As such, the antisense region of a siNA molecule of the invention can comprise sequence complementary to sequences that are unique to a particular allele to provide specificity in mediating selective RNAi against the disease, condition, or trait related allele.

25 In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a HIF1 gene, wherein the siNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule. In another embodiment, the siNA molecule is a
30 double stranded nucleic acid molecule, where each strand is about 21 nucleotides long and where about 19 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule, wherein at

least two 3' terminal nucleotides of each fragment of the siNA molecule are not base-paired to the nucleotides of the other fragment of the siNA molecule. In another embodiment, the siNA molecule is a double stranded nucleic acid molecule, where each strand is about 19 nucleotide long and where the nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule to form at least about 15 (e.g., 15, 16, 17, 18, or 19) base pairs, wherein one or both ends of the siNA molecule are blunt ends. In one embodiment, each of the two 3' terminal nucleotides of each fragment of the siNA molecule is a 2'-deoxypyrimidine nucleotide, such as a 2'-deoxy-thymidine. In another embodiment, all nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule. In another embodiment, the siNA molecule is a double stranded nucleic acid molecule of about 19 to about 25 base pairs having a sense region and an antisense region, where about 19 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the HIF1 gene. In another embodiment, about 21 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the HIF1 gene. In any of the above embodiments, the 5'-end of the fragment comprising said antisense region can optionally include a phosphate group.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the expression of a HIF1 RNA sequence (e.g., wherein said target RNA sequence is encoded by a HIF1 gene or a gene involved in the HIF1 pathway), wherein the siNA molecule does not contain any ribonucleotides and wherein each strand of the double-stranded siNA molecule is about 15 to about 30 nucleotides. In one embodiment, the siNA molecule is 21 nucleotides in length. Examples of non-ribonucleotide containing siNA constructs are combinations of stabilization chemistries shown in Table IV in any combination of Sense/Antisense chemistries, such as Stab 7/8, Stab 7/11, Stab 8/8, Stab 18/8, Stab 18/11, Stab 12/13, Stab 7/13, Stab 18/13, Stab 7/19, Stab 8/19, Stab 18/19, Stab 7/20, Stab 8/20, or Stab 18/20 (e.g., any siNA having Stab 7, 8, 11, 12, 13, 14, 15, 17, 18, 19, or 20 sense or antisense strands or any combination thereof).

In one embodiment, the invention features a chemically synthesized double stranded RNA molecule that directs cleavage of a HIF1 RNA via RNA interference, wherein each strand of said RNA molecule is about 15 to about 30 nucleotides in length;

one strand of the RNA molecule comprises nucleotide sequence having sufficient complementarity to the HIF1 RNA for the RNA molecule to direct cleavage of the HIF1 RNA via RNA interference; and wherein at least one strand of the RNA molecule optionally comprises one or more chemically modified nucleotides described herein, such as without limitation deoxynucleotides, 2'-O-methyl nucleotides, 2'-deoxy-2'-fluoro nucleotides, 2'-O-methoxyethyl nucleotides etc.

In one embodiment, the invention features a medicament comprising a siNA molecule of the invention.

In one embodiment, the invention features an active ingredient comprising a siNA molecule of the invention.

In one embodiment, the invention features the use of a double-stranded short interfering nucleic acid (siNA) molecule to inhibit, down-regulate, or reduce expression of a HIF1 gene, wherein the siNA molecule comprises one or more chemical modifications and each strand of the double-stranded siNA is independently about 15 to about 30 or more (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 or more) nucleotides long. In one embodiment, the siNA molecule of the invention is a double stranded nucleic acid molecule comprising one or more chemical modifications, where each of the two fragments of the siNA molecule independently comprise about 15 to about 40 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 23, 33, 34, 35, 36, 37, 38, 39, or 40) nucleotides and where one of the strands comprises at least 15 nucleotides that are complementary to nucleotide sequence of HIF1 encoding RNA or a portion thereof. In a non-limiting example, each of the two fragments of the siNA molecule comprise about 21 nucleotides. In another embodiment, the siNA molecule is a double stranded nucleic acid molecule comprising one or more chemical modifications, where each strand is about 21 nucleotide long and where about 19 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule, wherein at least two 3' terminal nucleotides of each fragment of the siNA molecule are not base-paired to the nucleotides of the other fragment of the siNA molecule. In another embodiment, the siNA molecule is a double stranded nucleic acid molecule comprising one or more chemical modifications, where each strand is about 19 nucleotide long and where the nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides

of the other fragment of the siNA molecule to form at least about 15 (e.g., 15, 16, 17, 18, or 19) base pairs, wherein one or both ends of the siNA molecule are blunt ends. In one embodiment, each of the two 3' terminal nucleotides of each fragment of the siNA molecule is a 2'-deoxy-pyrimidine nucleotide, such as a 2'-deoxy-thymidine. In another embodiment, all nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule. In another embodiment, the siNA molecule is a double stranded nucleic acid molecule of about 19 to about 25 base pairs having a sense region and an antisense region and comprising one or more chemical modifications, where about 19 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the HIF1 gene. In another embodiment, about 21 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the HIF1 gene. In any of the above embodiments, the 5'-end of the fragment comprising said antisense region can optionally include a phosphate group.

In one embodiment, the invention features the use of a double-stranded short interfering nucleic acid (siNA) molecule that inhibits, down-regulates, or reduces expression of a HIF1 gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of HIF1 RNA or a portion thereof, the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits, down-regulates, or reduces expression of a HIF1 gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of HIF1 RNA or a portion thereof, wherein the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits, down-regulates, or reduces expression of a HIF1 gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of HIF1 RNA that encodes a protein or portion thereof, the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification. In one embodiment, each strand of the siNA molecule comprises about 15 to about 30 or more (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 or more) nucleotides, wherein each strand comprises at least about 15 nucleotides that are complementary to the nucleotides of the other strand. In one embodiment, the siNA molecule is assembled from two oligonucleotide fragments, wherein one fragment comprises the nucleotide sequence of the antisense strand of the siNA molecule and a second fragment comprises nucleotide sequence of the sense region of the siNA molecule. In one embodiment, the sense strand is connected to the antisense strand via a linker molecule, such as a polynucleotide linker or a non-nucleotide linker. In a further embodiment, the pyrimidine nucleotides present in the sense strand are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-deoxy purine nucleotides. In another embodiment, the pyrimidine nucleotides present in the sense strand are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-O-methyl purine nucleotides. In still another embodiment, the pyrimidine nucleotides present in the antisense strand are 2'-deoxy-2'-fluoro pyrimidine nucleotides and any purine nucleotides present in the antisense strand are 2'-deoxy purine nucleotides. In another embodiment, the antisense strand comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides and one or more 2'-O-methyl purine nucleotides. In another embodiment, the pyrimidine nucleotides present in the antisense strand are 2'-deoxy-2'-fluoro pyrimidine nucleotides and any purine nucleotides present in the antisense strand are 2'-O-methyl purine nucleotides. In a further embodiment the sense strand comprises a 3'-end and a 5'-end, wherein a terminal cap moiety (e.g., an inverted deoxy abasic moiety or inverted deoxy nucleotide moiety such as inverted thymidine) is present at the 5'-end, the 3'-end, or both of the 5' and 3' ends of the sense strand. In another embodiment, the antisense strand comprises a phosphorothioate internucleotide linkage at the 3' end of the antisense

strand. In another embodiment, the antisense strand comprises a glyceryl modification at the 3' end. In another embodiment, the 5'-end of the antisense strand optionally includes a phosphate group.

In any of the above-described embodiments of a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of a HIF1 gene, wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification, each of the two strands of the siNA molecule can comprise about 15 to about 30 or more (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 or more) nucleotides. In one embodiment, about 15 to about 30 or more (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 or more) nucleotides of each strand of the siNA molecule are base-paired to the complementary nucleotides of the other strand of the siNA molecule. In another embodiment, about 15 to about 30 or more (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 or more) nucleotides of each strand of the siNA molecule are base-paired to the complementary nucleotides of the other strand of the siNA molecule, wherein at least two 3' terminal nucleotides of each strand of the siNA molecule are not base-paired to the nucleotides of the other strand of the siNA molecule. In another embodiment, each of the two 3' terminal nucleotides of each fragment of the siNA molecule is a 2'-deoxy-pyrimidine, such as 2'-deoxy-thymidine. In one embodiment, each strand of the siNA molecule is base-paired to the complementary nucleotides of the other strand of the siNA molecule. In one embodiment, about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides of the antisense strand are base-paired to the nucleotide sequence of the HIF1 RNA or a portion thereof. In one embodiment, about 18 to about 25 (e.g., about 18, 19, 20, 21, 22, 23, 24, or 25) nucleotides of the antisense strand are base-paired to the nucleotide sequence of the HIF1 RNA or a portion thereof.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of a HIF1 gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of HIF1 RNA or a portion thereof, the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule

comprises a sugar modification, and wherein the 5'-end of the antisense strand optionally includes a phosphate group.

5 In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of a HIF1 gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of HIF1 RNA or a portion thereof, the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule
10 comprises a sugar modification, and wherein the nucleotide sequence or a portion thereof of the antisense strand is complementary to a nucleotide sequence of the untranslated region or a portion thereof of the HIF1 RNA.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of a HIF1 gene, wherein one of the
15 strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of HIF1 RNA or a portion thereof, wherein the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand, wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule
20 comprises a sugar modification, and wherein the nucleotide sequence of the antisense strand is complementary to a nucleotide sequence of the HIF1 or a portion thereof that is present in the HIF1 RNA.

In one embodiment, the invention features a composition comprising a siNA molecule of the invention in a pharmaceutically acceptable carrier or diluent.

25 In a non-limiting example, the introduction of chemically-modified nucleotides into nucleic acid molecules provides a powerful tool in overcoming potential limitations of *in vivo* stability and bioavailability inherent to native RNA molecules that are delivered exogenously. For example, the use of chemically-modified nucleic acid molecules can enable a lower dose of a particular nucleic acid molecule for a given
30 therapeutic effect since chemically-modified nucleic acid molecules tend to have a longer half-life in serum. Furthermore, certain chemical modifications can improve the bioavailability of nucleic acid molecules by targeting particular cells or tissues and/or

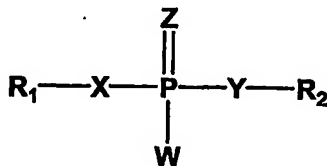
improving cellular uptake of the nucleic acid molecule. Therefore, even if the activity of a chemically-modified nucleic acid molecule is reduced as compared to a native nucleic acid molecule, for example, when compared to an all-RNA nucleic acid molecule, the overall activity of the modified nucleic acid molecule can be greater than that of the native molecule due to improved stability and/or delivery of the molecule. Unlike native unmodified siNA, chemically-modified siNA can also minimize the possibility of activating interferon activity in humans.

In any of the embodiments of siNA molecules described herein, the antisense region of a siNA molecule of the invention can comprise a phosphorothioate internucleotide linkage at the 3'-end of said antisense region. In any of the embodiments of siNA molecules described herein, the antisense region can comprise about one to about five phosphorothioate internucleotide linkages at the 5'-end of said antisense region. In any of the embodiments of siNA molecules described herein, the 3'-terminal nucleotide overhangs of a siNA molecule of the invention can comprise ribonucleotides or deoxyribonucleotides that are chemically-modified at a nucleic acid sugar, base, or backbone. In any of the embodiments of siNA molecules described herein, the 3'-terminal nucleotide overhangs can comprise one or more universal base ribonucleotides. In any of the embodiments of siNA molecules described herein, the 3'-terminal nucleotide overhangs can comprise one or more acyclic nucleotides.

One embodiment of the invention provides an expression vector comprising a nucleic acid sequence encoding at least one siNA molecule of the invention in a manner that allows expression of the nucleic acid molecule. Another embodiment of the invention provides a mammalian cell comprising such an expression vector. The mammalian cell can be a human cell. The siNA molecule of the expression vector can comprise a sense region and an antisense region. The antisense region can comprise sequence complementary to a RNA or DNA sequence encoding HIF1 and the sense region can comprise sequence complementary to the antisense region. The siNA molecule can comprise two distinct strands having complementary sense and antisense regions. The siNA molecule can comprise a single strand having complementary sense and antisense regions.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against

HIF1 inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides comprising a backbone modified internucleotide linkage having Formula I:

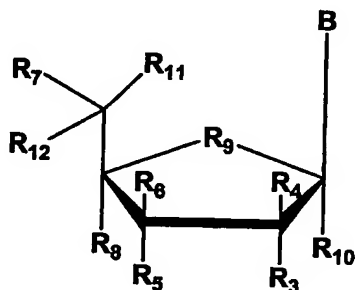


- 5 wherein each R1 and R2 is independently any nucleotide, non-nucleotide, or polynucleotide which can be naturally-occurring or chemically-modified, each X and Y is independently O, S, N, alkyl, or substituted alkyl, each Z and W is independently O, S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, aralkyl, or acetyl and wherein W, X, Y, and Z are optionally not all O. In another embodiment, a backbone modification of
- 10 the invention comprises a phosphonoacetate and/or thiophosphonoacetate internucleotide linkage (see for example Sheehan et al., 2003, *Nucleic Acids Research*, 31, 4109-4118).

- The chemically-modified internucleotide linkages having Formula I, for example, wherein any Z, W, X, and/or Y independently comprises a sulphur atom, can be present in one or both oligonucleotide strands of the siNA duplex, for example, in the sense
- 15 strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) chemically-modified internucleotide linkages having Formula I at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more
- 20 (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified internucleotide linkages having Formula I at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) pyrimidine nucleotides with chemically-modified internucleotide linkages having Formula I in the
- 25 sense strand, the antisense strand, or both strands. In yet another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) purine nucleotides with chemically-modified internucleotide linkages having Formula I in the sense strand, the antisense strand, or both strands. In another embodiment, a siNA molecule of the invention having

internucleotide linkage(s) of Formula I also comprises a chemically-modified nucleotide or non-nucleotide having any of Formulae I-VII.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against HIF1 inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides or non-nucleotides having Formula II:

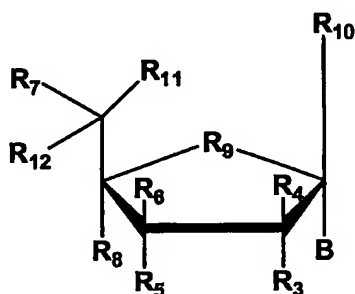


wherein each R3, R4, R5, R6, R7, R8, R10, R11 and R12 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, or group having Formula I or II; R9 is O, S, CH2, S=O, CHF, or CF2, and B is a nucleosidic base such as adenine, guanine, uracil, cytosine, thymine, 2-aminoadenosine, 5-methylcytosine, 2,6-diaminopurine, or any other non-naturally occurring base that can be complementary or non-complementary to target RNA or a non-nucleosidic base such as phenyl, naphthyl, 3-nitropyrrole, 5-nitroindole, nebularine, pyridone, pyridinone, or any other non-naturally occurring universal base that can be complementary or non-complementary to target RNA.

The chemically-modified nucleotide or non-nucleotide of Formula II can be present in one or both oligonucleotide strands of the siNA duplex, for example in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more chemically-modified nucleotides or non-nucleotides of Formula II at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the

invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified nucleotides or non-nucleotides of Formula II at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*,
 5 about 1, 2, 3, 4, 5, or more) chemically-modified nucleotides or non-nucleotides of Formula II at the 3'-end of the sense strand, the antisense strand, or both strands.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against HIF1 inside a cell or reconstituted *in vitro* system, wherein the chemical modification
 10 comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides or non-nucleotides having Formula III:



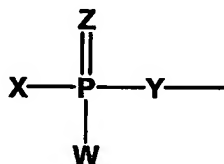
wherein each R3, R4, R5, R6, R7, R8, R10, R11 and R12 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl,
 15 N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, or group having Formula I or II; R9 is O, S, CH2, S=O, CHF, or CF2,
 20 and B is a nucleosidic base such as adenine, guanine, uracil, cytosine, thymine, 2-aminoadenosine, 5-methylcytosine, 2,6-diaminopurine, or any other non-naturally occurring base that can be employed to be complementary or non-complementary to target RNA or a non-nucleosidic base such as phenyl, naphthyl, 3-nitropyrrole, 5-nitroindole, nebularine, pyridone, pyridinone, or any other non-naturally occurring
 25 universal base that can be complementary or non-complementary to target RNA.

The chemically-modified nucleotide or non-nucleotide of Formula III can be present in one or both oligonucleotide strands of the siNA duplex, for example, in the

sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more chemically-modified nucleotide or non-nucleotide of Formula III at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can
 5 comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified nucleotide(s) or non-nucleotide(s) of Formula III at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified nucleotide or non-nucleotide of
 10 Formula III at the 3'-end of the sense strand, the antisense strand, or both strands.

In another embodiment, a siNA molecule of the invention comprises a nucleotide having Formula II or III, wherein the nucleotide having Formula II or III is in an inverted configuration. For example, the nucleotide having Formula II or III is connected to the siNA construct in a 3'-3', 3'-2', 2'-3', or 5'-5' configuration, such as at the 3'-end, the 5'-
 15 end, or both of the 3' and 5'-ends of one or both siNA strands.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against HIF1 inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises a 5'-terminal phosphate group having Formula IV:



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wherein each X and Y is independently O, S, N, alkyl, substituted alkyl, or alkylhalo; wherein each Z and W is independently O, S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, aralkyl, alkylhalo, or acetyl; and wherein W, X, Y and Z are not all O.

In one embodiment, the invention features a siNA molecule having a 5'-terminal
 25 phosphate group having Formula IV on the target-complementary strand, for example, a strand complementary to a target RNA, wherein the siNA molecule comprises an all RNA siNA molecule. In another embodiment, the invention features a siNA molecule having a 5'-terminal phosphate group having Formula IV on the target-complementary

strand wherein the siNA molecule also comprises about 1 to about 3 (*e.g.*, about 1, 2, or 3) nucleotide 3'-terminal nucleotide overhangs having about 1 to about 4 (*e.g.*, about 1, 2, 3, or 4) deoxyribonucleotides on the 3'-end of one or both strands. In another embodiment, a 5'-terminal phosphate group having Formula IV is present on the target-
5 complementary strand of a siNA molecule of the invention, for example a siNA molecule having chemical modifications having any of Formulae I-VII.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against HIF1 inside a cell or reconstituted *in vitro* system, wherein the chemical modification
10 comprises one or more phosphorothioate internucleotide linkages. For example, in a non-limiting example, the invention features a chemically-modified short interfering nucleic acid (siNA) having about 1, 2, 3, 4, 5, 6, 7, 8 or more phosphorothioate internucleotide linkages in one siNA strand. In yet another embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) individually having
15 about 1, 2, 3, 4, 5, 6, 7, 8 or more phosphorothioate internucleotide linkages in both siNA strands. The phosphorothioate internucleotide linkages can be present in one or both oligonucleotide strands of the siNA duplex, for example in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more phosphorothioate internucleotide linkages at the 3'-end, the 5'-end, or both of the 3'- and
20 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) consecutive phosphorothioate internucleotide linkages at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*,
25 about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) pyrimidine phosphorothioate internucleotide linkages in the sense strand, the antisense strand, or both strands. In yet another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) purine phosphorothioate internucleotide linkages in the sense strand, the antisense strand, or both strands.

30 In one embodiment, the invention features a siNA molecule, wherein the sense strand comprises one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or about one or more

(e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 10 or more, specifically about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

15 In another embodiment, the invention features a siNA molecule, wherein the sense strand comprises about 1 to about 5, specifically about 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5, or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without about 1 to about 5 or more, for example about 1, 2, 3, 4, 5, or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

In one embodiment, the invention features a siNA molecule, wherein the antisense strand comprises one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or about one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 10 or more, specifically about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3' and 5'-ends, being present in the same or different strand.

In another embodiment, the invention features a siNA molecule, wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without about 1 to about 5, for example about 1, 2, 3, 4, 5 or more phosphorothioate

internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule having about 1 to about 5 or more (specifically about 1, 2, 3, 4, 5 or more) phosphorothioate internucleotide linkages in each strand of the siNA molecule.

In another embodiment, the invention features a siNA molecule comprising 2'-5' internucleotide linkages. The 2'-5' internucleotide linkage(s) can be at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of one or both siNA sequence strands. In addition, the 2'-5' internucleotide linkage(s) can be present at various other positions within one or both siNA sequence strands, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more including every internucleotide linkage of a pyrimidine nucleotide in one or both strands of the siNA molecule can comprise a 2'-5' internucleotide linkage, or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more including every internucleotide linkage of a purine nucleotide in one or both strands of the siNA molecule can comprise a 2'-5' internucleotide linkage.

In another embodiment, a chemically-modified siNA molecule of the invention comprises a duplex having two strands, one or both of which can be chemically-modified, wherein each strand is independently about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides in length, wherein the duplex has about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) base pairs, and wherein the chemical modification comprises a structure having any of Formulae I-VII. For example, an exemplary chemically-modified siNA molecule of the invention comprises a duplex having two strands, one or both of which can be chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein each strand consists of about 21 nucleotides, each having a 2-nucleotide 3'-terminal nucleotide overhang, and wherein the duplex has about 19 base pairs. In another embodiment, a siNA molecule of the invention comprises a single stranded hairpin structure, wherein the siNA is about 36 to about 70 (e.g., about 36, 40, 45, 50, 55, 60, 65, or 70) nucleotides in length having about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) base pairs, and wherein the siNA can include a chemical modification comprising a structure having any of Formulae I-VII or any combination thereof. For example, an

exemplary chemically-modified siNA molecule of the invention comprises a linear oligonucleotide having about 42 to about 50 (e.g., about 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides that is chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein the linear oligonucleotide forms a hairpin structure having about 19 to about 21 (e.g., 19, 20, or 21) base pairs and a 2-nucleotide 3'-terminal nucleotide overhang. In another embodiment, a linear hairpin siNA molecule of the invention contains a stem loop motif, wherein the loop portion of the siNA molecule is biodegradable. For example, a linear hairpin siNA molecule of the invention is designed such that degradation of the loop portion of the siNA molecule *in vivo* can generate a double-stranded siNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucleotides.

In another embodiment, a siNA molecule of the invention comprises a hairpin structure, wherein the siNA is about 25 to about 50 (e.g., about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides in length having about 3 to about 25 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) base pairs, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a linear oligonucleotide having about 25 to about 35 (e.g., about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35) nucleotides that is chemically-modified with one or more chemical modifications having any of Formulae I-VII or any combination thereof, wherein the linear oligonucleotide forms a hairpin structure having about 3 to about 25 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) base pairs and a 5'-terminal phosphate group that can be chemically modified as described herein (for example a 5'-terminal phosphate group having Formula IV). In another embodiment, a linear hairpin siNA molecule of the invention contains a stem loop motif, wherein the loop portion of the siNA molecule is biodegradable. In one embodiment, a linear hairpin siNA molecule of the invention comprises a loop portion comprising a non-nucleotide linker.

In another embodiment, a siNA molecule of the invention comprises an asymmetric hairpin structure, wherein the siNA is about 25 to about 50 (e.g., about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides in length having about 3 to about 25 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10,

11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) base pairs, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a linear oligonucleotide having about 25 to about 35 (*e.g.*, about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35) nucleotides that is chemically-modified with one or more chemical modifications having any of Formulae I-VII or any combination thereof, wherein the linear oligonucleotide forms an asymmetric hairpin structure having about 3 to about 25 (*e.g.*, about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) base pairs and a 5'-terminal phosphate group that can be chemically modified as described herein (for example a 5'-terminal phosphate group having Formula IV). In one embodiment, an asymmetric hairpin siNA molecule of the invention contains a stem loop motif, wherein the loop portion of the siNA molecule is biodegradable. In another embodiment, an asymmetric hairpin siNA molecule of the invention comprises a loop portion comprising a non-nucleotide linker.

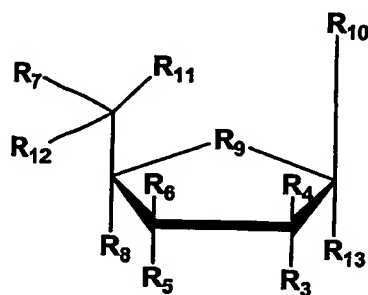
In another embodiment, a siNA molecule of the invention comprises an asymmetric double stranded structure having separate polynucleotide strands comprising sense and antisense regions, wherein the antisense region is about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides in length, wherein the sense region is about 3 to about 25 (*e.g.*, about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) nucleotides in length, wherein the sense region and the antisense region have at least 3 complementary nucleotides, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises an asymmetric double stranded structure having separate polynucleotide strands comprising sense and antisense regions, wherein the antisense region is about 18 to about 23 (*e.g.*, about 18, 19, 20, 21, 22, or 23) nucleotides in length and wherein the sense region is about 3 to about 15 (*e.g.*, about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15) nucleotides in length, wherein the sense region the antisense region have at least 3 complementary nucleotides, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. In another embodiment, the asymmetric double stranded siNA molecule can also have a 5'-terminal phosphate group that can be

chemically modified as described herein (for example a 5'-terminal phosphate group having Formula IV).

In another embodiment, a siNA molecule of the invention comprises a circular nucleic acid molecule, wherein the siNA is about 38 to about 70 (e.g., about 38, 40, 45, 50, 55, 60, 65, or 70) nucleotides in length having about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) base pairs, and wherein the siNA can include a chemical modification, which comprises a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a circular oligonucleotide having about 42 to about 50 (e.g., about 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides that is chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein the circular oligonucleotide forms a dumbbell shaped structure having about 19 base pairs and 2 loops.

In another embodiment, a circular siNA molecule of the invention contains two loop motifs, wherein one or both loop portions of the siNA molecule is biodegradable. For example, a circular siNA molecule of the invention is designed such that degradation of the loop portions of the siNA molecule *in vivo* can generate a double-stranded siNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucleotides.

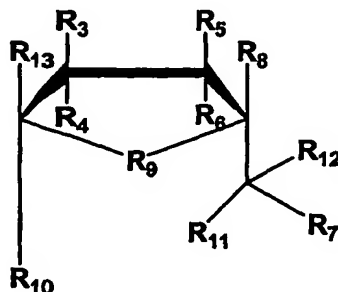
In one embodiment, a siNA molecule of the invention comprises at least one (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) abasic moiety, for example a compound having Formula V:



wherein each R3, R4, R5, R6, R7, R8, R10, R11, R12, and R13 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-

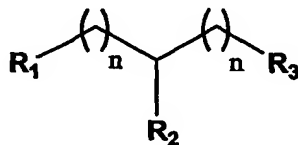
alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO₂, NO₂, N₃, NH₂, aminoalkyl, aminoacid, aminoacyl, ONH₂, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, or group having Formula I or II; R₉ is O, S, CH₂, S=O, CHF, or CF₂.

- 5 In one embodiment, a siNA molecule of the invention comprises at least one (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) inverted abasic moiety, for example a compound having Formula VI:



- wherein each R₃, R₄, R₅, R₆, R₇, R₈, R₁₀, R₁₁, R₁₂, and R₁₃ is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF₃, OCF₃, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO₂, NO₂, N₃, NH₂, aminoalkyl, aminoacid, aminoacyl, ONH₂, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, or group having Formula I or II; R₉ is O, S, CH₂, S=O, CHF, or CF₂, and either R₂, R₃, R₈ or R₁₃ serve as points of attachment to the siNA molecule of the invention.

- In another embodiment, a siNA molecule of the invention comprises at least one (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) substituted polyalkyl moieties, for example a compound having Formula VII:



wherein each *n* is independently an integer from 1 to 12, each R₁, R₂ and R₃ is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF₃,

OCF₃, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO₂, NO₂, N₃, NH₂, aminoalkyl, aminoacid, aminoacyl, ONH₂, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, or a group having Formula I, and R₁, R₂ or R₃ serves as points of attachment to the siNA molecule of the invention.

In another embodiment, the invention features a compound having Formula VII, wherein R₁ and R₂ are hydroxyl (OH) groups, n = 1, and R₃ comprises O and is the point of attachment to the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both strands of a double-stranded siNA molecule of the invention or to a single-stranded siNA molecule of the invention. This modification is referred to herein as "glyceryl" (for example modification 6 in Figure 10).

In another embodiment, a chemically modified nucleoside or non-nucleoside (e.g. a moiety having any of Formula V, VI or VII) of the invention is at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of a siNA molecule of the invention. For example, chemically modified nucleoside or non-nucleoside (e.g., a moiety having Formula V, VI or VII) can be present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense strand, the sense strand, or both antisense and sense strands of the siNA molecule. In one embodiment, the chemically modified nucleoside or non-nucleoside (e.g., a moiety having Formula V, VI or VII) is present at the 5'-end and 3'-end of the sense strand and the 3'-end of the antisense strand of a double stranded siNA molecule of the invention. In one embodiment, the chemically modified nucleoside or non-nucleoside (e.g., a moiety having Formula V, VI or VII) is present at the terminal position of the 5'-end and 3'-end of the sense strand and the 3'-end of the antisense strand of a double stranded siNA molecule of the invention. In one embodiment, the chemically modified nucleoside or non-nucleoside (e.g., a moiety having Formula V, VI or VII) is present at the two terminal positions of the 5'-end and 3'-end of the sense strand and the 3'-end of the antisense strand of a double stranded siNA molecule of the invention. In one embodiment, the chemically modified nucleoside or non-nucleoside (e.g., a moiety having Formula V, VI or VII) is present at the penultimate position of the 5'-end and 3'-end of the sense strand and the 3'-end of the antisense strand of a double stranded siNA molecule of the invention. In addition, a moiety having Formula VII can be present at the 3'-end or the 5'-end of a hairpin siNA molecule as described herein.

In another embodiment, a siNA molecule of the invention comprises an abasic residue having Formula V or VI, wherein the abasic residue having Formula VI or VI is connected to the siNA construct in a 3'-3', 3'-2', 2'-3', or 5'-5' configuration, such as at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both siNA strands.

5 In one embodiment, a siNA molecule of the invention comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) locked nucleic acid (LNA) nucleotides, for example, at the 5'-end, the 3'-end, both of the 5' and 3'-ends, or any combination thereof, of the siNA molecule.

10 In another embodiment, a siNA molecule of the invention comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) acyclic nucleotides, for example, at the 5'-end, the 3'-end, both of the 5' and 3'-ends, or any combination thereof, of the siNA molecule.

15 In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising a sense region, wherein any (*e.g.*, one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (*e.g.*, one or more or all) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (*e.g.*,
20 wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides).

25 In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising a sense region, wherein any (*e.g.*, one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (*e.g.*, one or more or all) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (*e.g.*,
30 wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides), wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said sense region are 2'-deoxy nucleotides.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising a sense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the sense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides).

10 In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising a sense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), wherein any (e.g., one or more or all) purine nucleotides present in the sense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), and wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said sense region are 2'-deoxy nucleotides.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising an antisense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides).

30 In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising an antisense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the antisense region are

2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), wherein any (e.g., one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), and wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said antisense region are 2'-deoxy nucleotides.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising an antisense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the antisense region are 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides).

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising an antisense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides).

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention capable of mediating RNA interference (RNAi) against HIF1 inside a cell or reconstituted *in vitro* system comprising a sense region, wherein one or more pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine

nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides), and an antisense region, wherein one or
5 more pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides
10 are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides). The sense region and/or the antisense region can have a terminal cap modification, such as any modification described herein or shown in Figure 10, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense and/or antisense sequence. The sense and/or antisense region can optionally
15 further comprise a 3'-terminal nucleotide overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides. The overhang nucleotides can further comprise one or more (e.g., about 1, 2, 3, 4 or more) phosphorothioate, phosphonoacetate, and/or thiophosphonoacetate internucleotide linkages. Non-limiting examples of these chemically-modified siNAs are shown in Figures 4 and 5 and Tables III and IV herein.

20 In any of these described embodiments, the purine nucleotides present in the sense region are alternatively 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides) and one or more purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all
25 purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides). Also, in any of these embodiments, one or more purine nucleotides present in the sense region are alternatively purine ribonucleotides (e.g., wherein all purine nucleotides are purine ribonucleotides or alternately a plurality of purine nucleotides are purine ribonucleotides) and any purine
30 nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides). Additionally, in any of these embodiments, one or more purine nucleotides present in the sense region and/or present in the antisense region are alternatively selected from the group consisting of 2'-

deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides (e.g., wherein all purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides or alternately a plurality of purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides).

In another embodiment, any modified nucleotides present in the siNA molecules of the invention, preferably in the antisense strand of the siNA molecules of the invention, but also optionally in the sense and/or both antisense and sense strands, comprise modified nucleotides having properties or characteristics similar to naturally occurring ribonucleotides. For example, the invention features siNA molecules including modified nucleotides having a Northern conformation (e.g., Northern pseudorotation cycle, see for example Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag ed., 1984). As such, chemically modified nucleotides present in the siNA molecules of the invention, preferably in the antisense strand of the siNA molecules of the invention, but also optionally in the sense and/or both antisense and sense strands, are resistant to nuclease degradation while at the same time maintaining the capacity to mediate RNAi. Non-limiting examples of nucleotides having a northern configuration include locked nucleic acid (LNA) nucleotides (e.g., 2'-O, 4'-C-methylene-(D-ribofuranosyl) nucleotides); 2'-methoxyethoxy (MOE) nucleotides; 2'-methyl-thio-ethyl, 2'-deoxy-2'-fluoro nucleotides, 2'-deoxy-2'-chloro nucleotides, 2'-azido nucleotides, and 2'-O-methyl nucleotides.

In one embodiment, the sense strand of a double stranded siNA molecule of the invention comprises a terminal cap moiety, (see for example Figure 10) such as an inverted deoxyabaisc moiety, at the 3'-end, 5'-end, or both 3' and 5'-ends of the sense strand.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid molecule (siNA) capable of mediating RNA interference (RNAi) against HIF1 inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises a conjugate covalently attached to the chemically-modified siNA molecule. Non-limiting examples of conjugates contemplated by the invention include conjugates

and ligands described in Vargeese *et al.*, USSN 10/427,160, filed April 30, 2003, incorporated by reference herein in its entirety, including the drawings. In another embodiment, the conjugate is covalently attached to the chemically-modified siNA molecule via a biodegradable linker. In one embodiment, the conjugate molecule is
5 attached at the 3'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In another embodiment, the conjugate molecule is attached at the 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In yet another embodiment, the conjugate molecule is attached both the 3'-end and 5'-end of either the sense strand, the
10 antisense strand, or both strands of the chemically-modified siNA molecule, or any combination thereof. In one embodiment, a conjugate molecule of the invention comprises a molecule that facilitates delivery of a chemically-modified siNA molecule into a biological system, such as a cell. In another embodiment, the conjugate molecule attached to the chemically-modified siNA molecule is a polyethylene glycol, human
15 serum albumin, or a ligand for a cellular receptor that can mediate cellular uptake. Examples of specific conjugate molecules contemplated by the instant invention that can be attached to chemically-modified siNA molecules are described in Vargeese *et al.*, U.S. Serial No. 10/201,394, filed July 22, 2002 incorporated by reference herein. The type of conjugates used and the extent of conjugation of siNA molecules of the invention
20 can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of siNA constructs while at the same time maintaining the ability of the siNA to mediate RNAi activity. As such, one skilled in the art can screen siNA constructs that are modified with various conjugates to determine whether the siNA conjugate complex possesses improved properties while maintaining the ability to mediate RNAi, for
25 example in animal models as are generally known in the art.

In one embodiment, the invention features a short interfering nucleic acid (siNA) molecule of the invention, wherein the siNA further comprises a nucleotide, non-nucleotide, or mixed nucleotide/non-nucleotide linker that joins the sense region of the siNA to the antisense region of the siNA. In one embodiment, a nucleotide linker of the
30 invention can be a linker of ≥ 2 nucleotides in length, for example about 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides in length. In another embodiment, the nucleotide linker can be a nucleic acid aptamer. By "aptamer" or "nucleic acid aptamer" as used herein is meant a nucleic acid molecule that binds specifically to a target molecule wherein the nucleic

acid molecule has sequence that comprises a sequence recognized by the target molecule in its natural setting. Alternately, an aptamer can be a nucleic acid molecule that binds to a target molecule where the target molecule does not naturally bind to a nucleic acid. The target molecule can be any molecule of interest. For example, the aptamer can be used to bind to a ligand-binding domain of a protein, thereby preventing interaction of the naturally occurring ligand with the protein. This is a non-limiting example and those in the art will recognize that other embodiments can be readily generated using techniques generally known in the art. (See, for example, Gold *et al.*, 1995, *Annu. Rev. Biochem.*, 64, 763; Brody and Gold, 2000, *J. Biotechnol.*, 74, 5; Sun, 2000, *Curr. Opin. Mol. Ther.*, 2, 100; Kusser, 2000, *J. Biotechnol.*, 74, 27; Hermann and Patel, 2000, *Science*, 287, 820; and Jayasena, 1999, *Clinical Chemistry*, 45, 1628.)

In yet another embodiment, a non-nucleotide linker of the invention comprises abasic nucleotide, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, polyhydrocarbon, or other polymeric compounds (e.g. polyethylene glycols such as those having between 2 and 100 ethylene glycol units). Specific examples include those described by Seela and Kaiser, *Nucleic Acids Res.* 1990, 18:6353 and *Nucleic Acids Res.* 1987, 15:3113; Cload and Schepartz, *J. Am. Chem. Soc.* 1991, 113:6324; Richardson and Schepartz, *J. Am. Chem. Soc.* 1991, 113:5109; Ma *et al.*, *Nucleic Acids Res.* 1993, 21:2585 and *Biochemistry* 1993, 32:1751; Durand *et al.*, *Nucleic Acids Res.* 1990, 18:6353; McCurdy *et al.*, *Nucleosides & Nucleotides* 1991, 10:287; Ischke *et al.*, *Tetrahedron Lett.* 1993, 34:301; Ono *et al.*, *Biochemistry* 1991, 30:9914; Arnold *et al.*, International Publication No. WO 89/02439; Usman *et al.*, International Publication No. WO 95/06731; Dudycz *et al.*, International Publication No. WO 95/11910 and Ferentz and Verdine, *J. Am. Chem. Soc.* 1991, 113:4000, all hereby incorporated by reference herein. A "non-nucleotide" further means any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound can be abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine, for example at the C1 position of the sugar.

In one embodiment, the invention features a short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein one or both strands of the siNA molecule that are assembled from

two separate oligonucleotides do not comprise any ribonucleotides. For example, a siNA molecule can be assembled from a single oligonucleotide where the sense and antisense regions of the siNA comprise separate oligonucleotides that do not have any ribonucleotides (e.g., nucleotides having a 2'-OH group) present in the oligonucleotides.

5 In another example, a siNA molecule can be assembled from a single oligonucleotide where the sense and antisense regions of the siNA are linked or circularized by a nucleotide or non-nucleotide linker as described herein, wherein the oligonucleotide does not have any ribonucleotides (e.g., nucleotides having a 2'-OH group) present in the oligonucleotide. Applicant has surprisingly found that the presense of ribonucleotides

10 (e.g., nucleotides having a 2'-hydroxyl group) within the siNA molecule is not required or essential to support RNAi activity. As such, in one embodiment, all positions within the siNA can include chemically modified nucleotides and/or non-nucleotides such as nucleotides and or non-nucleotides having Formula I, II, III, IV, V, VI, or VII or any combination thereof to the extent that the ability of the siNA molecule to support RNAi

15 activity in a cell is maintained.

In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted *in vitro* system comprising a single stranded polynucleotide having complementarity to a target nucleic acid sequence. In another embodiment, the single stranded siNA molecule of the invention comprises a 5'-terminal phosphate group. In another embodiment, the single

20 stranded siNA molecule of the invention comprises a 5'-terminal phosphate group and a 3'-terminal phosphate group (e.g., a 2',3'-cyclic phosphate). In another embodiment, the single stranded siNA molecule of the invention comprises about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides. In yet

25 another embodiment, the single stranded siNA molecule of the invention comprises one or more chemically modified nucleotides or non-nucleotides described herein. For example, all the positions within the siNA molecule can include chemically-modified nucleotides such as nucleotides having any of Formulae I-VII, or any combination thereof to the extent that the ability of the siNA molecule to support RNAi activity in a

30 cell is maintained.

In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted *in vitro* system comprising a single stranded polynucleotide having complementarity to a target nucleic

acid sequence, wherein one or more pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), and a terminal cap modification, such as any modification described herein or shown in Figure 10, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence. The siNA optionally further comprises about 1 to about 4 or more (e.g., about 1, 2, 3, 4 or more) terminal 2'-deoxynucleotides at the 3'-end of the siNA molecule, wherein the terminal nucleotides can further comprise one or more (e.g., 1, 2, 3, 4 or more) phosphorothioate, phosphonoacetate, and/or thiophosphonoacetate internucleotide linkages, and wherein the siNA optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group. In any of these embodiments, any purine nucleotides present in the antisense region are alternatively 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides). Also, in any of these embodiments, any purine nucleotides present in the siNA (i.e., purine nucleotides present in the sense and/or antisense region) can alternatively be locked nucleic acid (LNA) nucleotides (e.g., wherein all purine nucleotides are LNA nucleotides or alternately a plurality of purine nucleotides are LNA nucleotides). Also, in any of these embodiments, any purine nucleotides present in the siNA are alternatively 2'-methoxyethyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-methoxyethyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-methoxyethyl purine nucleotides). In another embodiment, any modified nucleotides present in the single stranded siNA molecules of the invention comprise modified nucleotides having properties or characteristics similar to naturally occurring ribonucleotides. For example, the invention features siNA molecules including modified nucleotides having a Northern conformation (e.g., Northern pseudorotation cycle, see for example Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag ed., 1984). As such, chemically modified nucleotides present in the single stranded siNA molecules of the invention are preferably resistant to nuclease degradation while at the same time maintaining the capacity to mediate RNAi.

In one embodiment, a siNA molecule of the invention comprises chemically modified nucleotides or non-nucleotides (e.g., having any of Formulae I-VII, such as 2'-deoxy, 2'-deoxy-2'-fluoro, or 2'-O-methyl nucleotides) at alternating positions within one or more strands or regions of the siNA molecule. For example, such chemical modifications can be introduced at every other position of a RNA based siNA molecule, starting at either the first or second nucleotide from the 3'-end or 5'-end of the siNA. In a non-limiting example, a double stranded siNA molecule of the invention in which each strand of the siNA is 21 nucleotides in length is featured wherein positions 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21 of each strand are chemically modified (e.g., with compounds having any of Formulae I-VII, such as 2'-deoxy, 2'-deoxy-2'-fluoro, or 2'-O-methyl nucleotides). In another non-limiting example, a double stranded siNA molecule of the invention in which each strand of the siNA is 21 nucleotides in length is featured wherein positions 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 of each strand are chemically modified (e.g., with compounds having any of Formulae I-VII, such as 2'-deoxy, 2'-deoxy-2'-fluoro, or 2'-O-methyl nucleotides). Such siNA molecules can further comprise terminal cap moieties and/or backbone modifications as described herein.

In one embodiment, the invention features a method for modulating the expression of a HIF1 gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the HIF1 gene; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the HIF1 gene in the cell.

In one embodiment, the invention features a method for modulating the expression of a HIF1 gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the HIF1 gene and wherein the sense strand sequence of the siNA comprises a sequence identical or substantially similar to the sequence of the target RNA; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the HIF1 gene in the cell.

In another embodiment, the invention features a method for modulating the expression of more than one HIF1 gene within a cell comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA

strands comprises a sequence complementary to RNA of the HIF1 genes; and (b) introducing the siNA molecules into a cell under conditions suitable to modulate the expression of the HIF1 genes in the cell.

5 In another embodiment, the invention features a method for modulating the expression of two or more HIF1 genes within a cell comprising: (a) synthesizing one or more siNA molecules of the invention, which can be chemically-modified, wherein the siNA strands comprise sequences complementary to RNA of the HIF1 genes and wherein the sense strand sequences of the siNAs comprise sequences identical or substantially similar to the sequences of the target RNAs; and (b) introducing the siNA
10 molecules into a cell under conditions suitable to modulate the expression of the HIF1 genes in the cell.

In another embodiment, the invention features a method for modulating the expression of more than one HIF1 gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the
15 siNA strands comprises a sequence complementary to RNA of the HIF1 gene and wherein the sense strand sequence of the siNA comprises a sequence identical or substantially similar to the sequences of the target RNAs; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the HIF1 genes in the cell.

20 In one embodiment, siNA molecules of the invention are used as reagents in *ex vivo* applications. For example, siNA reagents are introduced into tissue or cells that are transplanted into a subject for therapeutic effect. The cells and/or tissue can be derived from an organism or subject that later receives the explant, or can be derived from another organism or subject prior to transplantation. The siNA molecules can be used to
25 modulate the expression of one or more genes in the cells or tissue, such that the cells or tissue obtain a desired phenotype or are able to perform a function when transplanted in vivo. In one embodiment, certain target cells from a patient are extracted. These extracted cells are contacted with siNAs targeting a specific nucleotide sequence within the cells under conditions suitable for uptake of the siNAs by these cells (e.g. using
30 delivery reagents such as cationic lipids, liposomes and the like or using techniques such as electroporation to facilitate the delivery of siNAs into cells). The cells are then reintroduced back into the same patient or other patients. In one embodiment, the

invention features a method of modulating the expression of a HIF1 gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the HIF1 gene; and (b) introducing the siNA molecule into a
5 cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the HIF1 gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the HIF1 gene in that organism.

10 In one embodiment, the invention features a method of modulating the expression of a HIF1 gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the HIF1 gene and wherein the sense
15 strand sequence of the siNA comprises a sequence identical or substantially similar to the sequence of the target RNA; and (b) introducing the siNA molecule into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the HIF1 gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the
20 expression of the HIF1 gene in that organism.

In another embodiment, the invention features a method of modulating the expression of more than one HIF1 gene in a tissue explant comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the HIF1 genes; and (b)
25 introducing the siNA molecules into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the HIF1 genes in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the HIF1 genes in that
30 organism.

In one embodiment, the invention features a method of modulating the expression of a HIF1 gene in a subject or organism comprising: (a) synthesizing a siNA molecule

of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the HIF1 gene; and (b) introducing the siNA molecule into the subject or organism under conditions suitable to modulate the expression of the HIF1 gene in the subject or organism. The level of HIF1 protein or
5 RNA can be determined using various methods well-known in the art.

In another embodiment, the invention features a method of modulating the expression of more than one HIF1 gene in a subject or organism comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the
10 HIF1 genes; and (b) introducing the siNA molecules into the subject or organism under conditions suitable to modulate the expression of the HIF1 genes in the subject or organism. The level of HIF1 protein or RNA can be determined as is known in the art.

In one embodiment, the invention features a method for modulating the expression of a HIF1 gene within a cell comprising: (a) synthesizing a siNA molecule of the
15 invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the HIF1 gene; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the HIF1 gene in the cell.

In another embodiment, the invention features a method for modulating the expression of more than one HIF1 gene within a cell comprising: (a) synthesizing siNA
20 molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the HIF1 gene; and (b) contacting the cell *in vitro* or *in vivo* with the siNA molecule under conditions suitable to modulate the expression of the HIF1 genes in the cell.

In one embodiment, the invention features a method of modulating the expression of a HIF1 gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single
25 stranded sequence having complementarity to RNA of the HIF1 gene; and (b) contacting a cell of the tissue explant derived from a particular subject or organism with the siNA molecule under conditions suitable to modulate the expression of the HIF1 gene in the
30 tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the subject or organism the tissue was derived from or into

another subject or organism under conditions suitable to modulate the expression of the HIF1 gene in that subject or organism.

5 In another embodiment, the invention features a method of modulating the expression of more than one HIF1 gene in a tissue explant comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the HIF1 gene; and (b) introducing the siNA molecules into a cell of the tissue explant derived from a particular subject or organism under conditions suitable to modulate the expression of the HIF1 genes in the tissue explant. In another embodiment, the method further
10 comprises introducing the tissue explant back into the subject or organism the tissue was derived from or into another subject or organism under conditions suitable to modulate the expression of the HIF1 genes in that subject or organism.

In one embodiment, the invention features a method of modulating the expression of a HIF1 gene in a subject or organism comprising: (a) synthesizing a siNA molecule
15 of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the HIF1 gene; and (b) introducing the siNA molecule into the subject or organism under conditions suitable to modulate the expression of the HIF1 gene in the subject or organism.

In another embodiment, the invention features a method of modulating the expression of more than one HIF1 gene in a subject or organism comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the HIF1 gene; and (b) introducing the siNA molecules into the subject or organism under conditions suitable to modulate the expression of the HIF1 genes in the subject or
25 organism.

In one embodiment, the invention features a method of modulating the expression of a HIF1 gene in a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate the expression of the HIF1 gene in the subject or organism.

30 In one embodiment, the invention features a method for treating or preventing cancer in a subject or organism comprising contacting the subject or organism with a

siNA molecule of the invention under conditions suitable to modulate the expression of the HIF1 gene in the subject or organism.

In one embodiment, the invention features a method for treating or preventing cancer, such as blood vessel tumors (haemangioblastomas); tumors in the adrenal glands; clear-cell kidney cancers; von Hippel-Lindau (VHL) disease; ovarian cancer; cancers of non-lymphoid parenchymal organs, including the heart, placenta, skeletal muscle and lung; breast cancer; cancers of the head and neck, including various lymphomas such as mantle cell lymphoma, non-Hodgkins lymphoma, adenoma, squamous cell carcinoma, laryngeal carcinoma; cancers of the retina; cancers of the esophagus; multiple myeloma; melanoma; colorectal cancer; lung cancer; bladder cancer; prostate cancer; glioblastoma or any other cancer associated with hypoxia inducible proliferation, in a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate the expression of the HIF1 gene in the subject or organism.

In one embodiment, the invention features a method for treating or preventing a proliferative disease or condition in a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate the expression of the HIF1 gene in the subject or organism.

In one embodiment, the invention features a method for treating or preventing an ocular disease or condition in a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate the expression of the HIF1 gene in the subject or organism.

In another embodiment, the invention features a method of modulating the expression of more than one HIF1 genes in a subject or organism comprising contacting the subject or organism with one or more siNA molecules of the invention under conditions suitable to modulate the expression of the HIF1 genes in the subject or organism.

The siNA molecules of the invention can be designed to down regulate or inhibit target (e.g., HIF1) gene expression through RNAi targeting of a variety of RNA molecules. In one embodiment, the siNA molecules of the invention are used to target various RNAs corresponding to a target gene. Non-limiting examples of such RNAs

include messenger RNA (mRNA), alternate RNA splice variants of target gene(s), post-transcriptionally modified RNA of target gene(s), pre-mRNA of target gene(s), and/or RNA templates. If alternate splicing produces a family of transcripts that are distinguished by usage of appropriate exons, the instant invention can be used to inhibit gene expression through the appropriate exons to specifically inhibit or to distinguish among the functions of gene family members. For example, a protein that contains an alternatively spliced transmembrane domain can be expressed in both membrane bound and secreted forms. Use of the invention to target the exon containing the transmembrane domain can be used to determine the functional consequences of pharmaceutical targeting of membrane bound as opposed to the secreted form of the protein. Non-limiting examples of applications of the invention relating to targeting these RNA molecules include therapeutic pharmaceutical applications, pharmaceutical discovery applications, molecular diagnostic and gene function applications, and gene mapping, for example using single nucleotide polymorphism mapping with siNA molecules of the invention. Such applications can be implemented using known gene sequences or from partial sequences available from an expressed sequence tag (EST).

In another embodiment, the siNA molecules of the invention are used to target conserved sequences corresponding to a gene family or gene families such as HIF1 family genes. As such, siNA molecules targeting multiple HIF1 targets can provide increased therapeutic effect. In addition, siNA can be used to characterize pathways of gene function in a variety of applications. For example, the present invention can be used to inhibit the activity of target gene(s) in a pathway to determine the function of uncharacterized gene(s) in gene function analysis, mRNA function analysis, or translational analysis. The invention can be used to determine potential target gene pathways involved in various diseases and conditions toward pharmaceutical development. The invention can be used to understand pathways of gene expression involved in, for example, cancer and/or other proliferative diseases and conditions.

In one embodiment, siNA molecule(s) and/or methods of the invention are used to down regulate the expression of gene(s) that encode RNA referred to by Genbank Accession, for example, HIF1 genes encoding RNA sequence(s) referred to herein by Genbank Accession number, for example, Genbank Accession Nos. shown in Table I.

In one embodiment, the invention features a method comprising: (a) generating a library of siNA constructs having a predetermined complexity; and (b) assaying the siNA constructs of (a) above, under conditions suitable to determine RNAi target sites within the target RNA sequence. In one embodiment, the siNA molecules of (a) have strands of a fixed length, for example, about 23 nucleotides in length. In another embodiment, the siNA molecules of (a) are of differing length, for example having strands of about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. In another embodiment, fragments of target RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNase protection assays, to determine the most suitable target site(s) within the target RNA sequence. The target RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for *in vitro* systems, and by cellular expression in *in vivo* systems.

In one embodiment, the invention features a method comprising: (a) generating a randomized library of siNA constructs having a predetermined complexity, such as of 4^N , where N represents the number of base paired nucleotides in each of the siNA construct strands (eg. for a siNA construct having 21 nucleotide sense and antisense strands with 19 base pairs, the complexity would be 4^{19}); and (b) assaying the siNA constructs of (a) above, under conditions suitable to determine RNAi target sites within the target HIF1 RNA sequence. In another embodiment, the siNA molecules of (a) have strands of a fixed length, for example about 23 nucleotides in length. In yet another embodiment, the siNA molecules of (a) are of differing length, for example having strands of about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described in Example 6 herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. In another embodiment, fragments of HIF1 RNA are analyzed for detectable levels of cleavage, for example, by gel electrophoresis, northern blot analysis, or RNase protection assays, to determine the most suitable target site(s) within the target HIF1 RNA sequence. The target HIF1 RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for *in vitro* systems, and by cellular expression in *in vivo* systems.

In another embodiment, the invention features a method comprising: (a) analyzing the sequence of a RNA target encoded by a target gene; (b) synthesizing one or more sets of siNA molecules having sequence complementary to one or more regions of the RNA of (a); and (c) assaying the siNA molecules of (b) under conditions suitable to determine RNAi targets within the target RNA sequence. In one embodiment, the siNA molecules of (b) have strands of a fixed length, for example about 23 nucleotides in length. In another embodiment, the siNA molecules of (b) are of differing length, for example having strands of about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. Fragments of target RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNase protection assays, to determine the most suitable target site(s) within the target RNA sequence. The target RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for *in vitro* systems, and by expression in *in vivo* systems.

By "target site" is meant a sequence within a target RNA that is "targeted" for cleavage mediated by a siNA construct which contains sequences within its antisense region that are complementary to the target sequence.

By "detectable level of cleavage" is meant cleavage of target RNA (and formation of cleaved product RNAs) to an extent sufficient to discern cleavage products above the background of RNAs produced by random degradation of the target RNA. Production of cleavage products from 1-5% of the target RNA is sufficient to detect above the background for most methods of detection.

In one embodiment, the invention features a composition comprising a siNA molecule of the invention, which can be chemically-modified, in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features a pharmaceutical composition comprising siNA molecules of the invention, which can be chemically-modified, targeting one or more genes in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features a method for diagnosing a disease or condition in a subject comprising administering to the subject a composition of the invention under conditions suitable for the diagnosis of the disease or

condition in the subject. In another embodiment, the invention features a method for treating or preventing a disease or condition in a subject, comprising administering to the subject a composition of the invention under conditions suitable for the treatment or prevention of the disease or condition in the subject, alone or in conjunction with one or more other therapeutic compounds. In another embodiment, the invention features a method for treating or preventing cancer in a subject comprising administering to the subject a composition of the invention under conditions suitable for the treatment or prevention of cancer in the subject. In another embodiment, the invention features a method for treating or preventing a proliferative disease or condition in a subject comprising administering to the subject a composition of the invention under conditions suitable for the treatment or prevention of the proliferative disease or condition in the subject. In another embodiment, the invention features a method for treating or preventing an ocular disease or condition in a subject comprising administering to the subject a composition of the invention under conditions suitable for the treatment or prevention of the ocular disease or condition in the subject.

In another embodiment, the invention features a method for validating a HIF1 gene target, comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of a HIF1 target gene; (b) introducing the siNA molecule into a cell, tissue, subject, or organism under conditions suitable for modulating expression of the HIF1 target gene in the cell, tissue, subject, or organism; and (c) determining the function of the gene by assaying for any phenotypic change in the cell, tissue, subject, or organism.

In another embodiment, the invention features a method for validating a HIF1 target comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of a HIF1 target gene; (b) introducing the siNA molecule into a biological system under conditions suitable for modulating expression of the HIF1 target gene in the biological system; and (c) determining the function of the gene by assaying for any phenotypic change in the biological system.

By "biological system" is meant, material, in a purified or unpurified form, from biological sources, including but not limited to human or animal, wherein the system

comprises the components required for RNAi activity. The term "biological system" includes, for example, a cell, tissue, subject, or organism, or extract thereof. The term biological system also includes reconstituted RNAi systems that can be used in an *in vitro* setting.

5 By "phenotypic change" is meant any detectable change to a cell that occurs in response to contact or treatment with a nucleic acid molecule of the invention (e.g., siNA). Such detectable changes include, but are not limited to, changes in shape, size, proliferation, motility, protein expression or RNA expression or other physical or chemical changes as can be assayed by methods known in the art. The detectable change
10 can also include expression of reporter genes/molecules such as Green Florescent Protein (GFP) or various tags that are used to identify an expressed protein or any other cellular component that can be assayed.

In one embodiment, the invention features a kit containing a siNA molecule of the invention, which can be chemically-modified, that can be used to modulate the
15 expression of a HIF1 target gene in a biological system, including, for example, in a cell, tissue, subject, or organism. In another embodiment, the invention features a kit containing more than one siNA molecule of the invention, which can be chemically-modified, that can be used to modulate the expression of more than one HIF1 target gene in a biological system, including, for example, in a cell, tissue, subject, or organism.

20 In one embodiment, the invention features a cell containing one or more siNA molecules of the invention, which can be chemically-modified. In another embodiment, the cell containing a siNA molecule of the invention is a mammalian cell. In yet another embodiment, the cell containing a siNA molecule of the invention is a human cell.

In one embodiment, the synthesis of a siNA molecule of the invention, which can
25 be chemically-modified, comprises: (a) synthesis of two complementary strands of the siNA molecule; (b) annealing the two complementary strands together under conditions suitable to obtain a double-stranded siNA molecule. In another embodiment, synthesis of the two complementary strands of the siNA molecule is by solid phase oligonucleotide synthesis. In yet another embodiment, synthesis of the two complementary strands of the
30 siNA molecule is by solid phase tandem oligonucleotide synthesis.

In one embodiment, the invention features a method for synthesizing a siNA duplex molecule comprising: (a) synthesizing a first oligonucleotide sequence strand of the siNA molecule, wherein the first oligonucleotide sequence strand comprises a cleavable linker molecule that can be used as a scaffold for the synthesis of the second oligonucleotide sequence strand of the siNA; (b) synthesizing the second oligonucleotide sequence strand of siNA on the scaffold of the first oligonucleotide sequence strand, wherein the second oligonucleotide sequence strand further comprises a chemical moiety than can be used to purify the siNA duplex; (c) cleaving the linker molecule of (a) under conditions suitable for the two siNA oligonucleotide strands to hybridize and form a stable duplex; and (d) purifying the siNA duplex utilizing the chemical moiety of the second oligonucleotide sequence strand. In one embodiment, cleavage of the linker molecule in (c) above takes place during deprotection of the oligonucleotide, for example, under hydrolysis conditions using an alkylamine base such as methylamine. In one embodiment, the method of synthesis comprises solid phase synthesis on a solid support such as controlled pore glass (CPG) or polystyrene, wherein the first sequence of (a) is synthesized on a cleavable linker, such as a succinyl linker, using the solid support as a scaffold. The cleavable linker in (a) used as a scaffold for synthesizing the second strand can comprise similar reactivity as the solid support derivatized linker, such that cleavage of the solid support derivatized linker and the cleavable linker of (a) takes place concomitantly. In another embodiment, the chemical moiety of (b) that can be used to isolate the attached oligonucleotide sequence comprises a trityl group, for example a dimethoxytrityl group, which can be employed in a trityl-on synthesis strategy as described herein. In yet another embodiment, the chemical moiety, such as a dimethoxytrityl group, is removed during purification, for example, using acidic conditions.

In a further embodiment, the method for siNA synthesis is a solution phase synthesis or hybrid phase synthesis wherein both strands of the siNA duplex are synthesized in tandem using a cleavable linker attached to the first sequence which acts a scaffold for synthesis of the second sequence. Cleavage of the linker under conditions suitable for hybridization of the separate siNA sequence strands results in formation of the double-stranded siNA molecule.

In another embodiment, the invention features a method for synthesizing a siNA duplex molecule comprising: (a) synthesizing one oligonucleotide sequence strand of

the siNA molecule, wherein the sequence comprises a cleavable linker molecule that can be used as a scaffold for the synthesis of another oligonucleotide sequence; (b) synthesizing a second oligonucleotide sequence having complementarity to the first sequence strand on the scaffold of (a), wherein the second sequence comprises the other strand of the double-stranded siNA molecule and wherein the second sequence further comprises a chemical moiety that can be used to isolate the attached oligonucleotide sequence; (c) purifying the product of (b) utilizing the chemical moiety of the second oligonucleotide sequence strand under conditions suitable for isolating the full-length sequence comprising both siNA oligonucleotide strands connected by the cleavable linker and under conditions suitable for the two siNA oligonucleotide strands to hybridize and form a stable duplex. In one embodiment, cleavage of the linker molecule in (c) above takes place during deprotection of the oligonucleotide, for example, under hydrolysis conditions. In another embodiment, cleavage of the linker molecule in (c) above takes place after deprotection of the oligonucleotide. In another embodiment, the method of synthesis comprises solid phase synthesis on a solid support such as controlled pore glass (CPG) or polystyrene, wherein the first sequence of (a) is synthesized on a cleavable linker, such as a succinyl linker, using the solid support as a scaffold. The cleavable linker in (a) used as a scaffold for synthesizing the second strand can comprise similar reactivity or differing reactivity as the solid support derivatized linker, such that cleavage of the solid support derivatized linker and the cleavable linker of (a) takes place either concomitantly or sequentially. In one embodiment, the chemical moiety of (b) that can be used to isolate the attached oligonucleotide sequence comprises a trityl group, for example a dimethoxytrityl group.

In another embodiment, the invention features a method for making a double-stranded siNA molecule in a single synthetic process comprising: (a) synthesizing an oligonucleotide having a first and a second sequence, wherein the first sequence is complementary to the second sequence, and the first oligonucleotide sequence is linked to the second sequence via a cleavable linker, and wherein a terminal 5'-protecting group, for example, a 5'-O-dimethoxytrityl group (5'-O-DMT) remains on the oligonucleotide having the second sequence; (b) deprotecting the oligonucleotide whereby the deprotection results in the cleavage of the linker joining the two oligonucleotide sequences; and (c) purifying the product of (b) under conditions suitable for isolating the double-stranded siNA molecule, for example using a trityl-on synthesis strategy as described herein.

In another embodiment, the method of synthesis of siNA molecules of the invention comprises the teachings of Scaringe *et al.*, US Patent Nos. 5,889,136; 6,008,400; and 6,111,086, incorporated by reference herein in their entirety.

5 In one embodiment, the invention features siNA constructs that mediate RNAi against HIF1, wherein the siNA construct comprises one or more chemical modifications, for example, one or more chemical modifications having any of Formulae I-VII or any combination thereof that increases the nuclease resistance of the siNA construct.

10 In another embodiment, the invention features a method for generating siNA molecules with increased nuclease resistance comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased nuclease resistance.

15 In another embodiment, the invention features a method for generating siNA molecules with improved toxicologic profiles (e.g., have attenuated or no immunostimulatory properties) comprising (a) introducing nucleotides having any of Formula I-VII (e.g., siNA motifs referred to in Table IV) or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved toxicologic profiles.

20 In another embodiment, the invention features a method for generating siNA molecules that do not stimulate an interferon response (e.g., no interferon response or attenuated interferon response) in a cell, subject, or organism, comprising (a) introducing nucleotides having any of Formula I-VII (e.g., siNA motifs referred to in Table IV) or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of
25 step (a) under conditions suitable for isolating siNA molecules that do not stimulate an interferon response.

By "improved toxicologic profile", is meant that the chemically modified siNA construct exhibits decreased toxicity in a cell, subject, or organism compared to an unmodified siNA or siNA molecule having fewer modifications or modifications that are
30 less effective in imparting improved toxicology. In a non-limiting example, siNA molecules with improved toxicologic profiles are associated with a decreased or

attenuated immunostimulatory response in a cell, subject, or organism compared to an unmodified siNA or siNA molecule having fewer modifications or modifications that are less effective in imparting improved toxicology. In one embodiment, a siNA molecule with an improved toxicological profile comprises no ribonucleotides. In one
5 embodiment, a siNA molecule with an improved toxicological profile comprises less than 5 ribonucleotides (e.g., 1, 2, 3, or 4 ribonucleotides). In one embodiment, a siNA molecule with an improved toxicological profile comprises Stab 7, Stab 8, Stab 11, Stab 12, Stab 13, Stab 16, Stab 17, Stab 18, Stab 19, Stab 20, Stab 23, Stab 24, Stab 25, Stab 26, Stab 27, Stab 28, Stab 29, Stab 30, Stab 31, Stab 32 or any combination thereof (see
10 Table IV). In one embodiment, the level of immunostimulatory response associated with a given siNA molecule can be measured as is known in the art, for example by determining the level of PKR/interferon response, proliferation, B-cell activation, and/or cytokine production in assays to quantitate the immunostimulatory response of particular siNA molecules (see, for example, Leifer *et al.*, 2003, *J Immunother.* 26, 313-9; and U.S.
15 Patent No. 5968909, incorporated in its entirety by reference).

In one embodiment, the invention features siNA constructs that mediate RNAi against HIF1, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the binding affinity between the sense and antisense strands of the siNA construct.

20 In another embodiment, the invention features a method for generating siNA molecules with increased binding affinity between the sense and antisense strands of the siNA molecule comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased binding
25 affinity between the sense and antisense strands of the siNA molecule.

In one embodiment, the invention features siNA constructs that mediate RNAi against HIF1, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the binding affinity between the antisense strand of the siNA construct and a complementary target RNA sequence within a cell.

30 In one embodiment, the invention features siNA constructs that mediate RNAi against HIF1, wherein the siNA construct comprises one or more chemical modifications

described herein that modulates the binding affinity between the antisense strand of the siNA construct and a complementary target DNA sequence within a cell.

5 In another embodiment, the invention features a method for generating siNA molecules with increased binding affinity between the antisense strand of the siNA molecule and a complementary target RNA sequence comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased binding affinity between the antisense strand of the siNA molecule and a complementary target RNA sequence.

10 In another embodiment, the invention features a method for generating siNA molecules with increased binding affinity between the antisense strand of the siNA molecule and a complementary target DNA sequence comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for
15 isolating siNA molecules having increased binding affinity between the antisense strand of the siNA molecule and a complementary target DNA sequence.

In one embodiment, the invention features siNA constructs that mediate RNAi against HIF1, wherein the siNA construct comprises one or more chemical modifications described herein that modulate the polymerase activity of a cellular polymerase capable of generating additional endogenous siNA molecules having sequence homology to the
20 chemically-modified siNA construct.

In another embodiment, the invention features a method for generating siNA molecules capable of mediating increased polymerase activity of a cellular polymerase capable of generating additional endogenous siNA molecules having sequence homology to a chemically-modified siNA molecule comprising (a) introducing nucleotides having
25 any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules capable of mediating increased polymerase activity of a cellular polymerase capable of generating additional endogenous siNA molecules having sequence homology to the
30 chemically-modified siNA molecule.

In one embodiment, the invention features chemically-modified siNA constructs that mediate RNAi against HIF1 in a cell, wherein the chemical modifications do not significantly effect the interaction of siNA with a target RNA molecule, DNA molecule and/or proteins or other factors that are essential for RNAi in a manner that would
5 decrease the efficacy of RNAi mediated by such siNA constructs.

In another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against HIF1 comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for
10 isolating siNA molecules having improved RNAi activity.

In yet another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against HIF1 target RNA comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable
15 for isolating siNA molecules having improved RNAi activity against the target RNA.

In yet another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against HIF1 target DNA comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable
20 for isolating siNA molecules having improved RNAi activity against the target DNA.

In one embodiment, the invention features siNA constructs that mediate RNAi against HIF1, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the cellular uptake of the siNA construct.

In another embodiment, the invention features a method for generating siNA
25 molecules against HIF1 with improved cellular uptake comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved cellular uptake.

In one embodiment, the invention features siNA constructs that mediate RNAi
30 against HIF1, wherein the siNA construct comprises one or more chemical modifications described herein that increases the bioavailability of the siNA construct, for example, by

attaching polymeric conjugates such as polyethyleneglycol or equivalent conjugates that improve the pharmacokinetics of the siNA construct, or by attaching conjugates that target specific tissue types or cell types *in vivo*. Non-limiting examples of such conjugates are described in Vargeese *et al.*, U.S. Serial No. 10/201,394 incorporated by
5 reference herein.

In one embodiment, the invention features a method for generating siNA molecules of the invention with improved bioavailability comprising (a) introducing a conjugate into the structure of a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved
10 bioavailability. Such conjugates can include ligands for cellular receptors, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; polyamines, such as spermine or
15 spermidine; and others.

In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein said second sequence is chemically
20 modified in a manner that it can no longer act as a guide sequence for efficiently mediating RNA interference and/or be recognized by cellular proteins that facilitate RNAi.

In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that comprises a first nucleotide sequence complementary
25 to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein the second sequence is designed or modified in a manner that prevents its entry into the RNAi pathway as a guide sequence or as a sequence that is complementary to a target nucleic acid (e.g., RNA) sequence. Such design or modifications are expected to enhance the activity of siNA and/or
30 improve the specificity of siNA molecules of the invention. These modifications are also expected to minimize any off-target effects and/or associated toxicity.

In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein said second sequence is incapable of acting as a guide sequence for mediating RNA interference.

In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein said second sequence does not have a terminal 5'-hydroxyl (5'-OH) or 5'-phosphate group.

In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein said second sequence comprises a terminal cap moiety at the 5'-end of said second sequence. In one embodiment, the terminal cap moiety comprises an inverted abasic, inverted deoxy abasic, inverted nucleotide moiety, a group shown in Figure 10, an alkyl or cycloalkyl group, a heterocycle, or any other group that prevents RNAi activity in which the second sequence serves as a guide sequence or template for RNAi.

In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein said second sequence comprises a terminal cap moiety at the 5'-end and 3'-end of said second sequence. In one embodiment, each terminal cap moiety individually comprises an inverted abasic, inverted deoxy abasic, inverted nucleotide moiety, a group shown in Figure 10, an alkyl or cycloalkyl group, a heterocycle, or any other group that prevents RNAi activity in which the second sequence serves as a guide sequence or template for RNAi.

In one embodiment, the invention features a method for generating siNA molecules of the invention with improved specificity for down regulating or inhibiting the expression of a target nucleic acid (e.g., a DNA or RNA such as a gene or its corresponding RNA), comprising (a) introducing one or more chemical modifications

into the structure of a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved specificity. In another embodiment, the chemical modification used to improve specificity comprises terminal cap modifications at the 5'-end, 3'-end, or both 5' and 3'-ends of the siNA molecule. The terminal cap modifications can comprise, for example, structures shown in Figure 10 (e.g. inverted deoxybasic moieties) or any other chemical modification that renders a portion of the siNA molecule (e.g. the sense strand) incapable of mediating RNA interference against an off target nucleic acid sequence. In a non-limiting example, a siNA molecule is designed such that only the antisense sequence of the siNA molecule can serve as a guide sequence for RISC mediated degradation of a corresponding target RNA sequence. This can be accomplished by rendering the sense sequence of the siNA inactive by introducing chemical modifications to the sense strand that preclude recognition of the sense strand as a guide sequence by RNAi machinery. In one embodiment, such chemical modifications comprise any chemical group at the 5'-end of the sense strand of the siNA, or any other group that serves to render the sense strand inactive as a guide sequence for mediating RNA interference. These modifications, for example, can result in a molecule where the 5'-end of the sense strand no longer has a free 5'-hydroxyl (5'-OH) or a free 5'-phosphate group (e.g., phosphate, diphosphate, triphosphate, cyclic phosphate etc.). Non-limiting examples of such siNA constructs are described herein, such as "Stab 9/10", "Stab 7/8", "Stab 7/19", "Stab 17/22", "Stab 23/24", "Stab 24/25", and "Stab 24/26" (e.g., any siNA having Stab 7, 9, 17, 23, or 24 sense strands) chemistries and variants thereof (see Table IV) wherein the 5'-end and 3'-end of the sense strand of the siNA do not comprise a hydroxyl group or phosphate group.

In one embodiment, the invention features a method for generating siNA molecules of the invention with improved specificity for down regulating or inhibiting the expression of a target nucleic acid (e.g., a DNA or RNA such as a gene or its corresponding RNA), comprising introducing one or more chemical modifications into the structure of a siNA molecule that prevent a strand or portion of the siNA molecule from acting as a template or guide sequence for RNAi activity. In one embodiment, the inactive strand or sense region of the siNA molecule is the sense strand or sense region of the siNA molecule, i.e. the strand or region of the siNA that does not have complementarity to the target nucleic acid sequence. In one embodiment, such chemical modifications comprise any chemical group at the 5'-end of the sense strand or region of

the siNA that does not comprise a 5'-hydroxyl (5'-OH) or 5'-phosphate group, or any other group that serves to render the sense strand or sense region inactive as a guide sequence for mediating RNA interference. Non-limiting examples of such siNA constructs are described herein, such as "Stab 9/10", "Stab 7/8", "Stab 7/19", "Stab 17/22", "Stab 23/24", "Stab 24/25", and "Stab 24/26" chemistries and variants thereof (see Table IV) wherein the 5'-end and 3'-end of the sense strand of the siNA do not comprise a hydroxyl group or phosphate group.

In one embodiment, the invention features a method for screening siNA molecules that are active in mediating RNA interference against a target nucleic acid sequence comprising (a) generating a plurality of unmodified siNA molecules, (b) screening the siNA molecules of step (a) under conditions suitable for isolating siNA molecules that are active in mediating RNA interference against the target nucleic acid sequence, and (c) introducing chemical modifications (e.g. chemical modifications as described herein or as otherwise known in the art) into the active siNA molecules of (b). In one embodiment, the method further comprises re-screening the chemically modified siNA molecules of step (c) under conditions suitable for isolating chemically modified siNA molecules that are active in mediating RNA interference against the target nucleic acid sequence.

In one embodiment, the invention features a method for screening chemically modified siNA molecules that are active in mediating RNA interference against a target nucleic acid sequence comprising (a) generating a plurality of chemically modified siNA molecules (e.g. siNA molecules as described herein or as otherwise known in the art), and (b) screening the siNA molecules of step (a) under conditions suitable for isolating chemically modified siNA molecules that are active in mediating RNA interference against the target nucleic acid sequence.

The term "ligand" refers to any compound or molecule, such as a drug, peptide, hormone, or neurotransmitter, that is capable of interacting with another compound, such as a receptor, either directly or indirectly. The receptor that interacts with a ligand can be present on the surface of a cell or can alternately be an intercellular receptor. Interaction of the ligand with the receptor can result in a biochemical reaction, or can simply be a physical interaction or association.

In another embodiment, the invention features a method for generating siNA molecules of the invention with improved bioavailability comprising (a) introducing an excipient formulation to a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability.

5 Such excipients include polymers such as cyclodextrins, lipids, cationic lipids, polyamines, phospholipids, nanoparticles, receptors, ligands, and others.

In another embodiment, the invention features a method for generating siNA molecules of the invention with improved bioavailability comprising (a) introducing nucleotides having any of Formulae I-VII or any combination thereof into a siNA
10 molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability.

In another embodiment, polyethylene glycol (PEG) can be covalently attached to siNA compounds of the present invention. The attached PEG can be any molecular weight, preferably from about 2,000 to about 50,000 daltons (Da).

15 The present invention can be used alone or as a component of a kit having at least one of the reagents necessary to carry out the *in vitro* or *in vivo* introduction of RNA to test samples and/or subjects. For example, preferred components of the kit include a siNA molecule of the invention and a vehicle that promotes introduction of the siNA into cells of interest as described herein (e.g., using lipids and other methods of transfection
20 known in the art, see for example Beigelman *et al.*, US 6,395,713). The kit can be used for target validation, such as in determining gene function and/or activity, or in drug optimization, and in drug discovery (see for example Usman *et al.*, USSN 60/402,996). Such a kit can also include instructions to allow a user of the kit to practice the invention.

The term "short interfering nucleic acid", "siNA", "short interfering RNA",
25 "siRNA", "short interfering nucleic acid molecule", "short interfering oligonucleotide molecule", or "chemically-modified short interfering nucleic acid molecule" as used herein refers to any nucleic acid molecule capable of inhibiting or down regulating gene expression or viral replication, for example by mediating RNA interference "RNAi" or gene silencing in a sequence-specific manner; see for example Zamore *et al.*, 2000, Cell,
30 101, 25-33; Bass, 2001, *Nature*, 411, 428-429; Elbashir *et al.*, 2001, *Nature*, 411, 494-498; and Kreutzer *et al.*, International PCT Publication No. WO 00/44895; Zernicka-Goetz *et al.*, International PCT Publication No. WO 01/36646; Fire, International PCT

Publication No. WO 99/32619; Plaetinck *et al.*, International PCT Publication No. WO 00/01846; Mello and Fire, International PCT Publication No. WO 01/29058; Deschamps-Depaillette, International PCT Publication No. WO 99/07409; and Li *et al.*, International PCT Publication No. WO 00/44914; Allshire, 2002, *Science*, 297, 1818-1819; Volpe *et al.*, 2002, *Science*, 297, 1833-1837; Jenuwein, 2002, *Science*, 297, 2215-2218; and Hall *et al.*, 2002, *Science*, 297, 2232-2237; Hutvagner and Zamore, 2002, *Science*, 297, 2056-60; McManus *et al.*, 2002, *RNA*, 8, 842-850; Reinhart *et al.*, 2002, *Gene & Dev.*, 16, 1616-1626; and Reinhart & Bartel, 2002, *Science*, 297, 1831). Non limiting examples of siNA molecules of the invention are shown in Figures 4-6, and Tables II and III herein. For example the siNA can be a double-stranded polynucleotide molecule comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siNA can be assembled from two separate oligonucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary (i.e. each strand comprises nucleotide sequence that is complementary to nucleotide sequence in the other strand; such as where the antisense strand and sense strand form a duplex or double stranded structure, for example wherein the double stranded region is about 15 to about 30, e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 base pairs; the antisense strand comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense strand comprises nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof (e.g., about 15 to about 25 or more nucleotides of the siNA molecule are complementary to the target nucleic acid or a portion thereof). Alternatively, the siNA is assembled from a single oligonucleotide, where the self-complementary sense and antisense regions of the siNA are linked by means of a nucleic acid based or non-nucleic acid-based linker(s). The siNA can be a polynucleotide with a duplex, asymmetric duplex, hairpin or asymmetric hairpin secondary structure, having self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a separate target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siNA can be a circular single-

stranded polynucleotide having two or more loop structures and a stem comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof, and wherein the circular polynucleotide can be processed either *in vivo* or *in vitro* to generate an active siNA molecule capable of mediating RNAi. The siNA can also comprise a single stranded polynucleotide having nucleotide sequence complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof (for example, where such siNA molecule does not require the presence within the siNA molecule of nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof), wherein the single stranded polynucleotide can further comprise a terminal phosphate group, such as a 5'-phosphate (see for example Martinez *et al.*, 2002, *Cell*, 110, 563-574 and Schwarz *et al.*, 2002, *Molecular Cell*, 10, 537-568), or 5',3'-diphosphate. In certain embodiments, the siNA molecule of the invention comprises separate sense and antisense sequences or regions, wherein the sense and antisense regions are covalently linked by nucleotide or non-nucleotide linkers molecules as is known in the art, or are alternately non-covalently linked by ionic interactions, hydrogen bonding, van der Waals interactions, hydrophobic interactions, and/or stacking interactions. In certain embodiments, the siNA molecules of the invention comprise nucleotide sequence that is complementary to nucleotide sequence of a target gene. In another embodiment, the siNA molecule of the invention interacts with nucleotide sequence of a target gene in a manner that causes inhibition of expression of the target gene. As used herein, siNA molecules need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides. In certain embodiments, the short interfering nucleic acid molecules of the invention lack 2'-hydroxy (2'-OH) containing nucleotides. Applicant describes in certain embodiments short interfering nucleic acids that do not require the presence of nucleotides having a 2'-hydroxy group for mediating RNAi and as such, short interfering nucleic acid molecules of the invention optionally do not include any ribonucleotides (e.g., nucleotides having a 2'-OH group). Such siNA molecules that do not require the presence of ribonucleotides within the siNA molecule to support RNAi can however have an attached linker or linkers or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. Optionally, siNA molecules can comprise

ribonucleotides at about 5, 10, 20, 30, 40, or 50% of the nucleotide positions. The modified short interfering nucleic acid molecules of the invention can also be referred to as short interfering modified oligonucleotides "siMON." As used herein, the term siNA is meant to be equivalent to other terms used to describe nucleic acid molecules that are capable of mediating sequence specific RNAi, for example short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), short hairpin RNA (shRNA), short interfering oligonucleotide, short interfering nucleic acid, short interfering modified oligonucleotide, chemically-modified siRNA, post-transcriptional gene silencing RNA (ptgsRNA), and others. In addition, as used herein, the term RNAi is meant to be equivalent to other terms used to describe sequence specific RNA interference, such as post transcriptional gene silencing, translational inhibition, or epigenetics. For example, siNA molecules of the invention can be used to epigenetically silence genes at both the post-transcriptional level or the pre-transcriptional level. In a non-limiting example, epigenetic regulation of gene expression by siNA molecules of the invention can result from siNA mediated modification of chromatin structure or methylation pattern to alter gene expression (see, for example, Verdel *et al.*, 2004, *Science*, 303, 672-676; Pal-Bhadra *et al.*, 2004, *Science*, 303, 669-672; Allshire, 2002, *Science*, 297, 1818-1819; Volpe *et al.*, 2002, *Science*, 297, 1833-1837; Jenuwein, 2002, *Science*, 297, 2215-2218; and Hall *et al.*, 2002, *Science*, 297, 2232-2237).

In one embodiment, a siNA molecule of the invention is a duplex forming oligonucleotide "DFO", (see for example Figures 14-15 and Vaish *et al.*, USSN 10/727,780 filed December 3, 2003 and International PCT Application No. US04/16390, filed May 24, 2004).

In one embodiment, a siNA molecule of the invention is a multifunctional siNA, (see for example Figures 16-21 and Jadhav *et al.*, USSN 60/543,480 filed February 10, 2004 and International PCT Application No. US04/16390, filed May 24, 2004). The multifunctional siNA of the invention can comprise sequence targeting, for example, two regions of HIF1 RNA (see for example target sequences in Tables II and III).

By "asymmetric hairpin" as used herein is meant a linear siNA molecule comprising an antisense region, a loop portion that can comprise nucleotides or non-nucleotides, and a sense region that comprises fewer nucleotides than the antisense region to the extent that the sense region has enough complementary nucleotides to base

pair with the antisense region and form a duplex with loop. For example, an asymmetric hairpin siNA molecule of the invention can comprise an antisense region having length sufficient to mediate RNAi in a cell or in vitro system (e.g. about 15 to about 30, or about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides) and a
5 loop region comprising about 4 to about 12 (e.g., about 4, 5, 6, 7, 8, 9, 10, 11, or 12) nucleotides, and a sense region having about 3 to about 25 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) nucleotides that are complementary to the antisense region. The asymmetric hairpin siNA molecule can also comprise a 5'-terminal phosphate group that can be chemically modified. The loop
10 portion of the asymmetric hairpin siNA molecule can comprise nucleotides, non-nucleotides, linker molecules, or conjugate molecules as described herein.

By "asymmetric duplex" as used herein is meant a siNA molecule having two separate strands comprising a sense region and an antisense region, wherein the sense region comprises fewer nucleotides than the antisense region to the extent that the sense
15 region has enough complementary nucleotides to base pair with the antisense region and form a duplex. For example, an asymmetric duplex siNA molecule of the invention can comprise an antisense region having length sufficient to mediate RNAi in a cell or in vitro system (e.g. about 15 to about 30, or about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides) and a sense region having about 3 to about 25 (e.g.,
20 about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) nucleotides that are complementary to the antisense region.

By "modulate" is meant that the expression of the gene, or level of RNA molecule or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits is up regulated or down regulated,
25 such that expression, level, or activity is greater than or less than that observed in the absence of the modulator. For example, the term "modulate" can mean "inhibit," but the use of the word "modulate" is not limited to this definition.

By "inhibit", "down-regulate", or "reduce", it is meant that the expression of the gene, or level of RNA molecules or equivalent RNA molecules encoding one or more
30 proteins or protein subunits, or activity of one or more proteins or protein subunits, is reduced below that observed in the absence of the nucleic acid molecules (e.g., siNA) of the invention. In one embodiment, inhibition, down-regulation or reduction with an siNA

molecule is below that level observed in the presence of an inactive or attenuated molecule. In another embodiment, inhibition, down-regulation, or reduction with siNA molecules is below that level observed in the presence of, for example, an siNA molecule with scrambled sequence or with mismatches. In another embodiment, inhibition, down-regulation, or reduction of gene expression with a nucleic acid molecule of the instant invention is greater in the presence of the nucleic acid molecule than in its absence. In one embodiment, inhibition, down regulation, or reduction of gene expression is associated with post transcriptional silencing, such as RNAi mediated cleavage of a target nucleic acid molecule (e.g. RNA) or inhibition of translation. In one embodiment, inhibition, down regulation, or reduction of gene expression is associated with pretranscriptional silencing.

By "gene", or "target gene", is meant a nucleic acid that encodes an RNA, for example, nucleic acid sequences including, but not limited to, structural genes encoding a polypeptide. A gene or target gene can also encode a functional RNA (fRNA) or non-coding RNA (ncRNA), such as small temporal RNA (stRNA), micro RNA (miRNA), small nuclear RNA (snRNA), short interfering RNA (siRNA), small nucleolar RNA (snoRNA), ribosomal RNA (rRNA), transfer RNA (tRNA) and precursor RNAs thereof. Such non-coding RNAs can serve as target nucleic acid molecules for siNA mediated RNA interference in modulating the activity of fRNA or ncRNA involved in functional or regulatory cellular processes. Abberant fRNA or ncRNA activity leading to disease can therefore be modulated by siNA molecules of the invention. siNA molecules targeting fRNA and ncRNA can also be used to manipulate or alter the genotype or phenotype of a subject, organism or cell, by intervening in cellular processes such as genetic imprinting, transcription, translation, or nucleic acid processing (e.g., transamination, methylation etc.). The target gene can be a gene derived from a cell, an endogenous gene, a transgene, or exogenous genes such as genes of a pathogen, for example a virus, which is present in the cell after infection thereof. The cell containing the target gene can be derived from or contained in any organism, for example a plant, animal, protozoan, virus, bacterium, or fungus. Non-limiting examples of plants include monocots, dicots, or gymnosperms. Non-limiting examples of animals include vertebrates or invertebrates. Non-limiting examples of fungi include molds or yeasts. For a review, see for example Snyder and Gerstein, 2003, *Science*, 300, 258-260.

By "non-canonical base pair" is meant any non-Watson Crick base pair, such as mismatches and/or wobble base pairs, including flipped mismatches, single hydrogen bond mismatches, trans-type mismatches, triple base interactions, and quadruple base interactions. Non-limiting examples of such non-canonical base pairs include, but are not limited to, AC reverse Hoogsteen, AC wobble, AU reverse Hoogsteen, GU wobble, AA N7 amino, CC 2-carbonyl-amino(H1)-N3-amino(H2), GA sheared, UC 4-carbonyl-amino, UU imino-carbonyl, AC reverse wobble, AU Hoogsteen, AU reverse Watson Crick, CG reverse Watson Crick, GC N3-amino-amino N3, AA N1-amino symmetric, AA N7-amino symmetric, GA N7-N1 amino-carbonyl, GA+ carbonyl-amino N7-N1, GG N1-carbonyl symmetric, GG N3-amino symmetric, CC carbonyl-amino symmetric, CC N3-amino symmetric, UU 2-carbonyl-imino symmetric, UU 4-carbonyl-imino symmetric, AA amino-N3, AA N1-amino, AC amino 2-carbonyl, AC N3-amino, AC N7-amino, AU amino-4-carbonyl, AU N1-imino, AU N3-imino, AU N7-imino, CC carbonyl-amino, GA amino-N1, GA amino-N7, GA carbonyl-amino, GA N3-amino, GC amino-N3, GC carbonyl-amino, GC N3-amino, GC N7-amino, GG amino-N7, GG carbonyl-imino, GG N7-amino, GU amino-2-carbonyl, GU carbonyl-imino, GU imino-2-carbonyl, GU N7-imino, psiU imino-2-carbonyl, UC 4-carbonyl-amino, UC imino-carbonyl, UU imino-4-carbonyl, AC C2-H-N3, GA carbonyl-C2-H, UU imino-4-carbonyl 2 carbonyl-C5-H, AC amino(A) N3(C)-carbonyl, GC imino amino-carbonyl, Gpsi imino-2-carbonyl amino-2-carbonyl, and GU imino amino-2-carbonyl base pairs.

By "hypoxia-inducible factor-1" or "HIF1" as used herein is meant, any HIF1 protein, peptide, or polypeptide having HIF1 activity, such as encoded by HIF1 Genbank Accession Nos. shown in Table I (e.g., HIF1-alpha and/or HIF1-beta). The term HIF1 also refers to nucleic acid sequences encoding any HIF1 protein, peptide, or polypeptide having HIF1 activity. The term "HIF1" is also meant to include other HIF1 encoding sequence, such as HIF1 isoforms or other hypoxia-inducible factors (e.g., HIF2, HIF3, etc.), mutant HIF1 genes, splice variants of HIF1 genes, and HIF1 gene polymorphisms.

By "homologous sequence" is meant, a nucleotide sequence that is shared by one or more polynucleotide sequences, such as genes, gene transcripts and/or non-coding polynucleotides. For example, a homologous sequence can be a nucleotide sequence that is shared by two or more genes encoding related but different proteins, such as different members of a gene family, different protein epitopes, different protein isoforms or completely divergent genes, such as a cytokine and its corresponding receptors. A

homologous sequence can be a nucleotide sequence that is shared by two or more non-coding polynucleotides, such as noncoding DNA or RNA, regulatory sequences, introns, and sites of transcriptional control or regulation. Homologous sequences can also include conserved sequence regions shared by more than one polynucleotide sequence.

- 5 Homology does not need to be perfect homology (e.g., 100%), as partially homologous sequences are also contemplated by the instant invention (e.g., 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80% etc.).

- 10 By "conserved sequence region" is meant, a nucleotide sequence of one or more regions in a polynucleotide does not vary significantly between generations or from one biological system, subject, or organism to another biological system, subject, or organism. The polynucleotide can include both coding and non-coding DNA and RNA.

- 15 By "sense region" is meant a nucleotide sequence of a siNA molecule having complementarity to an antisense region of the siNA molecule. In addition, the sense region of a siNA molecule can comprise a nucleic acid sequence having homology with a target nucleic acid sequence.

- 20 By "antisense region" is meant a nucleotide sequence of a siNA molecule having complementarity to a target nucleic acid sequence. In addition, the antisense region of a siNA molecule can optionally comprise a nucleic acid sequence having complementarity to a sense region of the siNA molecule.

By "target nucleic acid" is meant any nucleic acid sequence whose expression or activity is to be modulated. The target nucleic acid can be DNA or RNA.

- 25 By "complementarity" is meant that a nucleic acid can form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non-traditional types. In reference to the nucleic molecules of the present invention, the binding free energy for a nucleic acid molecule with its complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, e.g., RNAi activity. Determination of binding free energies for nucleic acid molecules is well known in the art (see, e.g., Turner *et al.*, 1987, *CSH Symp. Quant. Biol.* LII pp.123-133; Frier *et al.*, 30 1986, *Proc. Nat. Acad. Sci. USA* 83:9373-9377; Turner *et al.*, 1987, *J. Am. Chem. Soc.* 109:3783-3785). A percent complementarity indicates the percentage of contiguous

residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, or 10 nucleotides out of a total of 10 nucleotides in the first oligonucleotide being based paired to a second nucleic acid sequence having 10 nucleotides represents 50%, 60%, 70%, 80%, 90%, and 100% complementary respectively). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence. In one embodiment, a siNA molecule of the invention comprises about 15 to about 30 or more (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 or more) nucleotides that are complementary to one or more target nucleic acid molecules or a portion thereof.

In one embodiment, the siNA molecules of the invention are used to treat cancer or other proliferative diseases, disorders, and/or conditions in a subject or organism.

By "cancer" or "proliferative disease" is meant, any disease, condition, trait, genotype or phenotype characterized by unregulated cell growth or replication as is known in the art, including AIDS related cancers such as Kaposi's sarcoma; blood vessel tumors (haemangioblastomas); tumors in the adrenal glands; clear-cell kidney cancers; von Hippel-Lindau (VHL) disease, breast cancers; bone cancers such as Osteosarcoma, Chondrosarcomas, Ewing's sarcoma, Fibrosarcomas, Giant cell tumors, Adamantinomas, and Chordomas; Brain cancers such as Meningiomas, Glioblastomas, Lower-Grade Astrocytomas, Oligodendrocytomas, Pituitary Tumors, Schwannomas, and Metastatic brain cancers; cancers of the head and neck including various lymphomas such as mantle cell lymphoma, non-Hodgkins lymphoma, adenoma, squamous cell carcinoma, laryngeal carcinoma, gallbladder and bile duct cancers, cancers of the retina such as retinoblastoma, cancers of the esophagus, gastric cancers, multiple myeloma, ovarian cancer, uterine cancer, thyroid cancer, testicular cancer, endometrial cancer, melanoma, colorectal cancer, lung cancer, bladder cancer, prostate cancer, lung cancer (including non-small cell lung carcinoma), pancreatic cancer, sarcomas, Wilms' tumor, cervical cancer, head and neck cancer, skin cancers, nasopharyngeal carcinoma, liposarcoma, epithelial carcinoma, renal cell carcinoma, gallbladder adeno carcinoma, parotid adenocarcinoma, endometrial sarcoma, multidrug resistant cancers; and proliferative diseases and conditions, such as neovascularization associated with tumor angiogenesis, macular degeneration (e.g., wet/dry AMD), corneal neovascularization, diabetic retinopathy, neovascular glaucoma, myopic degeneration and other proliferative diseases

and conditions such as restenosis and polycystic kidney disease, and any other cancer or proliferative disease, condition, trait, genotype or phenotype that can respond to the modulation of disease related gene (e.g., "HIF1") expression in a cell or tissue, alone or in combination with other therapies.

- 5 By "ocular disease" or "ocular condition" as used herein is meant, any disease, condition, trait, genotype or phenotype of the eye and related structures, such as Cystoid Macular Edema, Asteroid Hyalosis, Pathological Myopia and Posterior Staphyloma, Toxocariasis (Ocular Larva Migrans), Retinal Vein Occlusion, Posterior Vitreous Detachment, Tractional Retinal Tears, Epiretinal Membrane, Diabetic Retinopathy,
- 10 Lattice Degeneration, Retinal Vein Occlusion, Retinal Artery Occlusion, Macular Degeneration (e.g., age related macular degeneration such as wet AMD or dry AMD), Toxoplasmosis, Choroidal Melanoma, Acquired Retinoschisis, Hollenhorst Plaque, Idiopathic Central Serous Chorioretinopathy, Macular Hole, Presumed Ocular Histoplasmosis Syndrome, Retinal Macroaneurysm, Retinitis Pigmentosa, Retinal
- 15 Detachment, Hypertensive Retinopathy, Retinal Pigment Epithelium (RPE) Detachment, Papillophlebitis, Ocular Ischemic Syndrome, Coats' Disease, Leber's Miliary Aneurysm, Conjunctival Neoplasms, Allergic Conjunctivitis, Vernal Conjunctivitis, Acute Bacterial Conjunctivitis, Allergic Conjunctivitis & Vernal Keratoconjunctivitis, Viral Conjunctivitis, Bacterial Conjunctivitis, Chlamydial & Gonococcal Conjunctivitis,
- 20 Conjunctival Laceration, Episcleritis, Scleritis, Pingueculitis, Pterygium, Superior Limbic Keratoconjunctivitis (SLK of Theodore), Toxic Conjunctivitis, Conjunctivitis with Pseudomembrane, Giant Papillary Conjunctivitis, Terrien's Marginal Degeneration, Acanthamoeba Keratitis, Fungal Keratitis, Filamentary Keratitis, Bacterial Keratitis, Keratitis Sicca/Dry Eye Syndrome, Bacterial Keratitis, Herpes Simplex Keratitis, Sterile
- 25 Corneal Infiltrates, Phlyctenulosis, Corneal Abrasion & Recurrent Corneal Erosion, Corneal Foreign Body, Chemical Burs, Epithelial Basement Membrane Dystrophy (EBMD), Thygeson's Superficial Punctate Keratopathy, Corneal Laceration, Salzmann's Nodular Degeneration, Fuchs' Endothelial Dystrophy, Crystalline Lens Subluxation, Ciliary-Block Glaucoma, Primary Open-Angle Glaucoma, Pigment Dispersion
- 30 Syndrome and Pigmentary Glaucoma, Pseudoexfoliation Syndrom and Pseudoexfoliative Glaucoma, Anterior Uveitis, Primary Open Angle Glaucoma, Uveitic Glaucoma & Glaucomatocyclitic Crisis, Pigment Dispersion Syndrome & Pigmentary Glaucoma, Acute Angle Closure Glaucoma, Anterior Uveitis, Hyphema, Angle Recession Glaucoma, Lens Induced Glaucoma, Pseudoexfoliation Syndrome and Pseudoexfoliative

Glaucoma, Axenfeld-Rieger Syndrome, Neovascular Glaucoma, Pars Planitis, Choroidal Rupture, Duane's Retraction Syndrome, Toxic/Nutritional Optic Neuropathy, Aberrant Regeneration of Cranial Nerve III, Intracranial Mass Lesions, Carotid-Cavernous Sinus Fistula, Anterior Ischemic Optic Neuropathy, Optic Disc Edema & Papilledema, Cranial Nerve III Palsy, Cranial Nerve IV Palsy, Cranial Nerve VI Palsy, Cranial Nerve VII (Facial Nerve) Palsy, Homer's Syndrome, Internuclear Ophthalmoplegia, Optic Nerve Head Hypoplasia, Optic Pit, Tonic Pupil, Optic Nerve Head Drusen, Demyelinating Optic Neuropathy (Optic Neuritis, Retrobulbar Optic Neuritis), Amaurosis Fugax and Transient Ischemic Attack, Pseudotumor Cerebri, Pituitary Adenoma, Molluscum Contagiosum, Canaliculitis, Verruca and Papilloma, Pediculosis and Phthiriasis, Blepharitis, Hordeolum, Preseptal Cellulitis, Chalazion, Basal Cell Carcinoma, Herpes Zoster Ophthalmicus, Pediculosis & Phthiriasis, Blow-out Fracture, Chronic Epiphora, Dacryocystitis, Herpes Simplex Blepharitis, Orbital Cellulitis, Senile Entropion, and Squamous Cell Carcinoma.

In one embodiment of the present invention, each sequence of a siRNA molecule of the invention is independently about 15 to about 30 nucleotides in length, in specific embodiments about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length. In another embodiment, the siRNA duplexes of the invention independently comprise about 15 to about 30 base pairs (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30). In another embodiment, one or more strands of the siRNA molecule of the invention independently comprises about 15 to about 30 nucleotides (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) that are complementary to a target nucleic acid molecule. In yet another embodiment, siRNA molecules of the invention comprising hairpin or circular structures are about 35 to about 55 (*e.g.*, about 35, 40, 45, 50 or 55) nucleotides in length, or about 38 to about 44 (*e.g.*, about 38, 39, 40, 41, 42, 43, or 44) nucleotides in length and comprising about 15 to about 25 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) base pairs. Exemplary siRNA molecules of the invention are shown in Table II. Exemplary synthetic siRNA molecules of the invention are shown in Table III and/or Figures 4-5.

As used herein "cell" is used in its usual biological sense, and does not refer to an entire multicellular organism, *e.g.*, specifically does not refer to a human. The cell can be present in an organism, *e.g.*, birds, plants and mammals such as humans, cows, sheep, apes, monkeys, swine, dogs, and cats. The cell can be prokaryotic (*e.g.*, bacterial cell) or

eukaryotic (e.g., mammalian or plant cell). The cell can be of somatic or germ line origin, totipotent or pluripotent, dividing or non-dividing. The cell can also be derived from or can comprise a gamete or embryo, a stem cell, or a fully differentiated cell.

5 The siNA molecules of the invention are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells or tissues. The nucleic acid or nucleic acid complexes can be locally administered to relevant tissues *ex vivo*, or *in vivo* through direct dermal application, transdermal application, or injection, with or without their incorporation in biopolymers. In particular embodiments, the nucleic acid molecules of the invention comprise sequences
10 shown in Tables II-III and/or Figures 4-5. Examples of such nucleic acid molecules consist essentially of sequences defined in these tables and figures. Furthermore, the chemically modified constructs described in Table IV can be applied to any siNA sequence of the invention.

In another aspect, the invention provides mammalian cells containing one or more
15 siNA molecules of this invention. The one or more siNA molecules can independently be targeted to the same or different sites.

By "RNA" is meant a molecule comprising at least one ribonucleotide residue. By "ribonucleotide" is meant a nucleotide with a hydroxyl group at the 2' position of a β -D-ribofuranose moiety. The terms include double-stranded RNA, single-stranded RNA,
20 isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the siNA or internally, for example at one or more nucleotides of the RNA.
25 Nucleotides in the RNA molecules of the instant invention can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of naturally-occurring RNA.

By "subject" is meant an organism, which is a donor or recipient of explanted cells
30 or the cells themselves. "Subject" also refers to an organism to which the nucleic acid molecules of the invention can be administered. A subject can be a mammal or mammalian cells, including a human or human cells.

The term "phosphorothioate" as used herein refers to an internucleotide linkage having Formula I, wherein Z and/or W comprise a sulfur atom. Hence, the term phosphorothioate refers to both phosphorothioate and phosphorodithioate internucleotide linkages.

5 The term "phosphonoacetate" as used herein refers to an internucleotide linkage having Formula I, wherein Z and/or W comprise an acetyl or protected acetyl group.

 The term "thiophosphonoacetate" as used herein refers to an internucleotide linkage having Formula I, wherein Z comprises an acetyl or protected acetyl group and W comprises a sulfur atom or alternately W comprises an acetyl or protected acetyl
10 group and Z comprises a sulfur atom.

 The term "universal base" as used herein refers to nucleotide base analogs that form base pairs with each of the natural DNA/RNA bases with little discrimination between them. Non-limiting examples of universal bases include C-phenyl, C-naphthyl and other aromatic derivatives, inosine, azole carboxamides, and nitroazole derivatives
15 such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole as known in the art (see for example Loakes, 2001, *Nucleic Acids Research*, 29, 2437-2447).

 The term "acyclic nucleotide" as used herein refers to any nucleotide having an acyclic ribose sugar, for example where any of the ribose carbons (C1, C2, C3, C4, or C5), are independently or in combination absent from the nucleotide.

20 The nucleic acid molecules of the instant invention, individually, or in combination or in conjunction with other drugs, can be used to for preventing or treating cancer, proliferative, or ocular diseases and conditions in a subject or organism.

 For example, the siNA molecules can be administered to a subject or can be administered to other appropriate cells evident to those skilled in the art, individually or
25 in combination with one or more drugs under conditions suitable for the treatment.

 In a further embodiment, the siNA molecules can be used in combination with other known treatments to prevent or treat cancer, proliferative, or ocular diseases and conditions in a subject or organism. For example, the described molecules could be used in combination with one or more known compounds, treatments, or procedures to

prevent or treat cancer, proliferative, or ocular diseases and conditions in a subject or organism as are known in the art.

In one embodiment, the invention features an expression vector comprising a nucleic acid sequence encoding at least one siNA molecule of the invention, in a manner
5 which allows expression of the siNA molecule. For example, the vector can contain sequence(s) encoding both strands of a siNA molecule comprising a duplex. The vector can also contain sequence(s) encoding a single nucleic acid molecule that is self-complementary and thus forms a siNA molecule. Non-limiting examples of such expression vectors are described in Paul *et al.*, 2002, *Nature Biotechnology*, 19, 505;
10 Miyagishi and Taira, 2002, *Nature Biotechnology*, 19, 497; Lee *et al.*, 2002, *Nature Biotechnology*, 19, 500; and Novina *et al.*, 2002, *Nature Medicine*, advance online publication doi:10.1038/nm725.

In another embodiment, the invention features a mammalian cell, for example, a human cell, including an expression vector of the invention.

15 In yet another embodiment, the expression vector of the invention comprises a sequence for a siNA molecule having complementarity to a RNA molecule referred to by a Genbank Accession numbers, for example Genbank Accession Nos. shown in Table I.

In one embodiment, an expression vector of the invention comprises a nucleic acid sequence encoding two or more siNA molecules, which can be the same or different.

20 In another aspect of the invention, siNA molecules that interact with target RNA molecules and down-regulate gene encoding target RNA molecules (for example target RNA molecules referred to by Genbank Accession numbers herein) are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. siNA expressing viral vectors can be constructed based
25 on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. The recombinant vectors capable of expressing the siNA molecules can be delivered as described herein, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of siNA molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the siNA molecules bind and down-regulate
30 gene function or expression via RNA interference (RNAi). Delivery of siNA expressing vectors can be systemic, such as by intravenous or intramuscular administration, by

administration to target cells ex-planted from a subject followed by reintroduction into the subject, or by any other means that would allow for introduction into the desired target cell.

By "vectors" is meant any nucleic acid- and/or viral-based technique used to
5 deliver a desired nucleic acid.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a non-limiting example of a scheme for the synthesis of siNA
10 molecules. The complementary siNA sequence strands, strand 1 and strand 2, are synthesized in tandem and are connected by a cleavable linkage, such as a nucleotide succinate or abasic succinate, which can be the same or different from the cleavable linker used for solid phase synthesis on a solid support. The synthesis can be either solid phase or solution phase, in the example shown, the synthesis is a solid phase synthesis.
15 The synthesis is performed such that a protecting group, such as a dimethoxytrityl group, remains intact on the terminal nucleotide of the tandem oligonucleotide. Upon cleavage and deprotection of the oligonucleotide, the two siNA strands spontaneously hybridize to form a siNA duplex, which allows the purification of the duplex by utilizing the properties of the terminal protecting group, for example by applying a trityl on
20 purification method wherein only duplexes/oligonucleotides with the terminal protecting group are isolated.

Figure 2 shows a MALDI-TOF mass spectrum of a purified siNA duplex synthesized by a method of the invention. The two peaks shown correspond to the predicted mass of the separate siNA sequence strands. This result demonstrates that the
25 siNA duplex generated from tandem synthesis can be purified as a single entity using a simple trityl-on purification methodology.

Figure 3 shows a non-limiting proposed mechanistic representation of target RNA degradation involved in RNAi. Double-stranded RNA (dsRNA), which is generated by RNA-dependent RNA polymerase (RdRP) from foreign single-stranded RNA, for
30 example viral, transposon, or other exogenous RNA, activates the DICER enzyme that in turn generates siNA duplexes. Alternately, synthetic or expressed siNA can be

introduced directly into a cell by appropriate means. An active siNA complex forms which recognizes a target RNA, resulting in degradation of the target RNA by the RISC endonuclease complex or in the synthesis of additional RNA by RNA-dependent RNA polymerase (RdRP), which can activate DICER and result in additional siNA molecules, thereby amplifying the RNAi response.

Figure 4A-F shows non-limiting examples of chemically-modified siNA constructs of the present invention. In the figure, N stands for any nucleotide (adenosine, guanosine, cytosine, uridine, or optionally thymidine, for example thymidine can be substituted in the overhanging regions designated by parenthesis (N N). Various modifications are shown for the sense and antisense strands of the siNA constructs.

Figure 4A: The sense strand comprises 21 nucleotides wherein the two terminal 3'-nucleotides are optionally base paired and wherein all nucleotides present are ribonucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all nucleotides present are ribonucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the antisense strand.

Figure 4B: The sense strand comprises 21 nucleotides wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-O-methyl modified nucleotides

except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the sense and antisense strand.

Figure 4C: The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-O-methyl or 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the antisense strand.

Figure 4D: The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein and wherein all purine nucleotides that may be present are 2'-deoxy nucleotides. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate,

phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the antisense strand.

Figure 4E: The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the antisense strand.

Figure 4F: The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein and wherein all purine nucleotides that may be present are 2'-deoxy nucleotides. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and having one 3'-terminal phosphorothioate internucleotide linkage and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-deoxy nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the antisense strand. The antisense strand of constructs A-F comprise sequence complementary to any target nucleic acid sequence of

the invention. Furthermore, when a glyceryl moiety (L) is present at the 3'-end of the antisense strand for any construct shown in Figure 4 A-F, the modified internucleotide linkage is optional.

Figure 5A-F shows non-limiting examples of specific chemically-modified siNA sequences of the invention. A-F applies the chemical modifications described in Figure 4A-F to a HIF1 siNA sequence. Such chemical modifications can be applied to any HIF1 sequence and/or HIF1 polymorphism sequence.

Figure 6 shows non-limiting examples of different siNA constructs of the invention. The examples shown (constructs 1, 2, and 3) have 19 representative base pairs; however, different embodiments of the invention include any number of base pairs described herein. Bracketed regions represent nucleotide overhangs, for example, comprising about 1, 2, 3, or 4 nucleotides in length, preferably about 2 nucleotides. Constructs 1 and 2 can be used independently for RNAi activity. Construct 2 can comprise a polynucleotide or non-nucleotide linker, which can optionally be designed as a biodegradable linker. In one embodiment, the loop structure shown in construct 2 can comprise a biodegradable linker that results in the formation of construct 1 *in vivo* and/or *in vitro*. In another example, construct 3 can be used to generate construct 2 under the same principle wherein a linker is used to generate the active siNA construct 2 *in vivo* and/or *in vitro*, which can optionally utilize another biodegradable linker to generate the active siNA construct 1 *in vivo* and/or *in vitro*. As such, the stability and/or activity of the siNA constructs can be modulated based on the design of the siNA construct for use *in vivo* or *in vitro* and/or *in vitro*.

Figure 7A-C is a diagrammatic representation of a scheme utilized in generating an expression cassette to generate siNA hairpin constructs.

Figure 7A: A DNA oligomer is synthesized with a 5'-restriction site (R1) sequence followed by a region having sequence identical (sense region of siNA) to a predetermined HIF1 target sequence, wherein the sense region comprises, for example, about 19, 20, 21, or 22 nucleotides (N) in length, which is followed by a loop sequence of defined sequence (X), comprising, for example, about 3 to about 10 nucleotides.

Figure 7B: The synthetic construct is then extended by DNA polymerase to generate a hairpin structure having self-complementary sequence that will result in a

siNA transcript having specificity for a HIF1 target sequence and having self-complementary sense and antisense regions.

5 **Figure 7C:** The construct is heated (for example to about 95°C) to linearize the sequence, thus allowing extension of a complementary second DNA strand using a primer to the 3'-restriction sequence of the first strand. The double-stranded DNA is then inserted into an appropriate vector for expression in cells. The construct can be designed such that a 3'-terminal nucleotide overhang results from the transcription, for example, by engineering restriction sites and/or utilizing a poly-U termination region as described in Paul *et al.*, 2002, *Nature Biotechnology*, 29, 505-508.

10 **Figure 8A-C** is a diagrammatic representation of a scheme utilized in generating an expression cassette to generate double-stranded siNA constructs.

15 **Figure 8A:** A DNA oligomer is synthesized with a 5'-restriction (R1) site sequence followed by a region having sequence identical (sense region of siNA) to a predetermined HIF1 target sequence, wherein the sense region comprises, for example, about 19, 20, 21, or 22 nucleotides (N) in length, and which is followed by a 3'-restriction site (R2) which is adjacent to a loop sequence of defined sequence (X).

Figure 8B: The synthetic construct is then extended by DNA polymerase to generate a hairpin structure having self-complementary sequence.

20 **Figure 8C:** The construct is processed by restriction enzymes specific to R1 and R2 to generate a double-stranded DNA which is then inserted into an appropriate vector for expression in cells. The transcription cassette is designed such that a U6 promoter region flanks each side of the dsDNA which generates the separate sense and antisense strands of the siNA. Poly T termination sequences can be added to the constructs to generate U overhangs in the resulting transcript.

25 **Figure 9A-E** is a diagrammatic representation of a method used to determine target sites for siNA mediated RNAi within a particular target nucleic acid sequence, such as messenger RNA.

Figure 9A: A pool of siNA oligonucleotides are synthesized wherein the antisense region of the siNA constructs has complementarity to target sites across the target

nucleic acid sequence, and wherein the sense region comprises sequence complementary to the antisense region of the siNA.

Figure 9B&C: (Figure 9B) The sequences are pooled and are inserted into vectors such that (Figure 9C) transfection of a vector into cells results in the expression
5 of the siNA.

Figure 9D: Cells are sorted based on phenotypic change that is associated with modulation of the target nucleic acid sequence.

Figure 9E: The siNA is isolated from the sorted cells and is sequenced to identify efficacious target sites within the target nucleic acid sequence.

10 **Figure 10** shows non-limiting examples of different stabilization chemistries (1-10) that can be used, for example, to stabilize the 3'-end of siNA sequences of the invention, including (1) [3-3']-inverted deoxyribose; (2) deoxyribonucleotide; (3) [5'-3']-3'-deoxyribonucleotide; (4) [5'-3']-ribonucleotide; (5) [5'-3']-3'-O-methyl ribonucleotide; (6) 3'-glyceryl; (7) [3'-5']-3'-deoxyribonucleotide; (8) [3'-3']-deoxyribonucleotide; (9) [5'-
15 2']-deoxyribonucleotide; and (10) [5-3']-dideoxyribonucleotide. In addition to modified and unmodified backbone chemistries indicated in the figure, these chemistries can be combined with different backbone modifications as described herein, for example, backbone modifications having Formula I. In addition, the 2'-deoxy nucleotide shown 5' to the terminal modifications shown can be another modified or unmodified nucleotide
20 or non-nucleotide described herein, for example modifications having any of Formulae I-VII or any combination thereof.

Figure 11 shows a non-limiting example of a strategy used to identify chemically modified siNA constructs of the invention that are nuclease resistance while preserving the ability to mediate RNAi activity. Chemical modifications are introduced into the
25 siNA construct based on educated design parameters (e.g. introducing 2'-modifications, base modifications, backbone modifications, terminal cap modifications etc). The modified construct is tested in an appropriate system (e.g. human serum for nuclease resistance, shown, or an animal model for PK/delivery parameters). In parallel, the siNA construct is tested for RNAi activity, for example in a cell culture system such as a
30 luciferase reporter assay). Lead siNA constructs are then identified which possess a particular characteristic while maintaining RNAi activity, and can be further modified

and assayed once again. This same approach can be used to identify siNA-conjugate molecules with improved pharmacokinetic profiles, delivery, and RNAi activity.

Figure 12 shows non-limiting examples of phosphorylated siNA molecules of the invention, including linear and duplex constructs and asymmetric derivatives thereof.

5 Figure 13 shows non-limiting examples of chemically modified terminal phosphate groups of the invention.

10 Figure 14A shows a non-limiting example of methodology used to design self complementary DFO constructs utilizing palidrome and/or repeat nucleic acid sequences that are identified in a target nucleic acid sequence. (i) A palidrome or repeat sequence is identified in a nucleic acid target sequence. (ii) A sequence is designed that is complementary to the target nucleic acid sequence and the palidrome sequence. (iii) An inverse repeat sequence of the non-palidrome/repeat portion of the complementary sequence is appended to the 3'-end of the complementary sequence to generate a self complementary DFO molecule comprising sequence complementary to the nucleic acid target. (iv) The DFO molecule can self-assemble to form a double stranded oligonucleotide. Figure 14B shows a non-limiting representative example of a duplex forming oligonucleotide sequence. Figure 14C shows a non-limiting example of the self assembly schematic of a representative duplex forming oligonucleotide sequence. Figure 14D shows a non-limiting example of the self assembly schematic of a representative duplex forming oligonucleotide sequence followed by interaction with a target nucleic acid sequence resulting in modulation of gene expression.

20 Figure 15 shows a non-limiting example of the design of self complementary DFO constructs utilizing palidrome and/or repeat nucleic acid sequences that are incorporated into the DFO constructs that have sequence complementary to any target nucleic acid sequence of interest. Incorporation of these palidrome/repeat sequences allow the design of DFO constructs that form duplexes in which each strand is capable of mediating modulation of target gene expression, for example by RNAi. First, the target sequence is identified. A complementary sequence is then generated in which nucleotide or non-nucleotide modifications (shown as X or Y) are introduced into the complementary sequence that generate an artificial palidrome (shown as XYXYXY in the Figure). An inverse repeat of the non-palidrome/repeat complementary sequence is appended to the 3'-end of the complementary sequence to generate a self complementary

DFO comprising sequence complementary to the nucleic acid target. The DFO can self-assemble to form a double stranded oligonucleotide.

Figure 16 shows non-limiting examples of multifunctional siNA molecules of the invention comprising two separate polynucleotide sequences that are each capable of mediating RNAi directed cleavage of differing target nucleic acid sequences. Figure 16A shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 3'-ends of each polynucleotide sequence in the multifunctional siNA. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. Figure 16B shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 5'-ends of each polynucleotide sequence in the multifunctional siNA. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences.

Figure 17 shows non-limiting examples of multifunctional siNA molecules of the invention comprising a single polynucleotide sequence comprising distinct regions that are each capable of mediating RNAi directed cleavage of differing target nucleic acid sequences. Figure 17A shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the second complementary region is situated at the 3'-end of the polynucleotide sequence in the multifunctional siNA. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. Figure 17B shows a non-limiting example of a multifunctional siNA

molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first complementary region is situated at the 5'-end of the polynucleotide sequence in the multifunctional siNA. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. In one embodiment, these multifunctional siNA constructs are processed in vivo or in vitro to generate multifunctional siNA constructs as shown in Figure 16.

Figure 18 shows non-limiting examples of multifunctional siNA molecules of the invention comprising two separate polynucleotide sequences that are each capable of mediating RNAi directed cleavage of differing target nucleic acid sequences and wherein the multifunctional siNA construct further comprises a self complementary, palindrome, or repeat region, thus enabling shorter bifunctional siNA constructs that can mediate RNA interference against differing target nucleic acid sequences. Figure 18A shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 3'-ends of each polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary regions further comprise a self complementary, palindrome, or repeat region. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. Figure 18B shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 5'-ends of each polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary regions further comprise a self complementary, palindrome, or repeat region. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences.

Figure 19 shows non-limiting examples of multifunctional siNA molecules of the invention comprising a single polynucleotide sequence comprising distinct regions that are each capable of mediating RNAi directed cleavage of differing target nucleic acid sequences and wherein the multifunctional siNA construct further comprises a self complementary, palindrome, or repeat region, thus enabling shorter bifunctional siNA constructs that can mediate RNA interference against differing target nucleic acid sequences. Figure 19A shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the second complementary region is situated at the 3'-end of the polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary regions further comprise a self complementary, palindrome, or repeat region. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. Figure 19B shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first complementary region is situated at the 5'-end of the polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary regions further comprise a self complementary, palindrome, or repeat region. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. In one embodiment, these multifunctional siNA constructs are processed in vivo or in vitro to generate multifunctional siNA constructs as shown in Figure 18.

Figure 20 shows a non-limiting example of how multifunctional siNA molecules of the invention can target two separate target nucleic acid molecules, such as separate RNA molecules encoding differing proteins, for example, a cytokine and its corresponding receptor, differing viral strains, a virus and a cellular protein involved in viral infection or replication, or differing proteins involved in a common or divergent biologic pathway that is implicated in the maintenance of progression of disease. Each strand of the multifunctional siNA construct comprises a region having complementarity

to separate target nucleic acid molecules. The multifunctional siNA molecule is designed such that each strand of the siNA can be utilized by the RISC complex to initiate RNA interference mediated cleavage of its corresponding target. These design parameters can include destabilization of each end of the siNA construct (see for
5 example Schwarz *et al.*, 2003, *Cell*, 115, 199-208). Such destabilization can be accomplished for example by using guanosine-cytidine base pairs, alternate base pairs (e.g., wobbles), or destabilizing chemically modified nucleotides at terminal nucleotide positions as is known in the art.

Figure 21 shows a non-limiting example of how multifunctional siNA molecules
10 of the invention can target two separate target nucleic acid sequences within the same target nucleic acid molecule, such as alternate coding regions of a RNA, coding and non-coding regions of a RNA, or alternate splice variant regions of a RNA. Each strand of the multifunctional siNA construct comprises a region having complementarity to the separate regions of the target nucleic acid molecule. The multifunctional siNA molecule
15 is designed such that each strand of the siNA can be utilized by the RISC complex to initiate RNA interference mediated cleavage of its corresponding target region. These design parameters can include destabilization of each end of the siNA construct (see for example Schwarz *et al.*, 2003, *Cell*, 115, 199-208). Such destabilization can be accomplished for example by using guanosine-cytidine base pairs, alternate base pairs
20 (e.g., wobbles), or destabilizing chemically modified nucleotides at terminal nucleotide positions as is known in the art.

Figure 22 shows a non-limiting example of reduction of HIF1 mRNA in A549 cells mediated by chemically modified siNAs that target HIF1 mRNA. A549 cells were transfected with 0.25 ug/well of lipid complexed with 25 nM siNA. Active stabilized
25 siNA constructs (see Tables III and IV) were compared to untreated cells, a matched chemistry irrelevant siNA control construct (IC), and cells transfected with lipid alone (transfection control). As shown in the figure, the siNA constructs significantly reduce HIF1 RNA expression.

DETAILED DESCRIPTION OF THE INVENTION

30 Mechanism of Action of Nucleic Acid Molecules of the Invention

The discussion that follows discusses the proposed mechanism of RNA interference mediated by short interfering RNA as is presently known, and is not meant to be limiting and is not an admission of prior art. Applicant demonstrates herein that chemically-modified short interfering nucleic acids possess similar or improved capacity to mediate RNAi as do siRNA molecules and are expected to possess improved stability and activity *in vivo*; therefore, this discussion is not meant to be limiting only to siRNA and can be applied to siNA as a whole. By "improved capacity to mediate RNAi" or "improved RNAi activity" is meant to include RNAi activity measured *in vitro* and/or *in vivo* where the RNAi activity is a reflection of both the ability of the siNA to mediate RNAi and the stability of the siNAs of the invention. In this invention, the product of these activities can be increased *in vitro* and/or *in vivo* compared to an all RNA siRNA or a siNA containing a plurality of ribonucleotides. In some cases, the activity or stability of the siNA molecule can be decreased (i.e., less than ten-fold), but the overall activity of the siNA molecule is enhanced *in vitro* and/or *in vivo*.

RNA interference refers to the process of sequence specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Fire *et al.*, 1998, *Nature*, 391, 806). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes which is commonly shared by diverse flora and phyla (Fire *et al.*, 1999, *Trends Genet.*, 15, 358). Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response through a mechanism that has yet to be fully characterized. This mechanism appears to be different from the interferon response that results from dsRNA-mediated activation of protein kinase PKR and 2', 5'-oligoadenylate synthetase resulting in non-specific cleavage of mRNA by ribonuclease L.

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as Dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs) (Bernstein *et al.*, 2001,

Nature, 409, 363). Short interfering RNAs derived from Dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes. Dicer has also been implicated in the excision of 21- and 22-nucleotide small temporal RNAs (stRNAs) from precursor RNA of conserved structure that are implicated in translational control (Hutvagner *et al.*, 2001, *Science*, 293, 834). The RNAi response also features an endonuclease complex containing a siRNA, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence homologous to the siRNA. Cleavage of the target RNA takes place in the middle of the region complementary to the guide sequence of the siRNA duplex (Elbashir *et al.*, 2001, *Genes Dev.*, 15, 188). In addition, RNA interference can also involve small RNA (e.g., micro-RNA or miRNA) mediated gene silencing, presumably through cellular mechanisms that regulate chromatin structure and thereby prevent transcription of target gene sequences (see for example Allshire, 2002, *Science*, 297, 1818-1819; Volpe *et al.*, 2002, *Science*, 297, 1833-1837; Jenuwein, 2002, *Science*, 297, 2215-2218; and Hall *et al.*, 2002, *Science*, 297, 2232-2237). As such, siRNA molecules of the invention can be used to mediate gene silencing via interaction with RNA transcripts or alternately by interaction with particular gene sequences, wherein such interaction results in gene silencing either at the transcriptional level or post-transcriptional level.

RNAi has been studied in a variety of systems. Fire *et al.*, 1998, *Nature*, 391, 806, were the first to observe RNAi in *C. elegans*. Wianny and Goetz, 1999, *Nature Cell Biol.*, 2, 70, describe RNAi mediated by dsRNA in mouse embryos. Hammond *et al.*, 2000, *Nature*, 404, 293, describe RNAi in *Drosophila* cells transfected with dsRNA. Elbashir *et al.*, 2001, *Nature*, 411, 494, describe RNAi induced by introduction of duplexes of synthetic 21-nucleotide RNAs in cultured mammalian cells including human embryonic kidney and HeLa cells. Recent work in *Drosophila* embryonic lysates has revealed certain requirements for siRNA length, structure, chemical composition, and sequence that are essential to mediate efficient RNAi activity. These studies have shown that 21 nucleotide siRNA duplexes are most active when containing two 2-nucleotide 3'-terminal nucleotide overhangs. Furthermore, substitution of one or both siRNA strands with 2'-deoxy or 2'-O-methyl nucleotides abolishes RNAi activity, whereas substitution of 3'-terminal siRNA nucleotides with deoxy nucleotides was shown to be tolerated. Mismatch sequences in the center of the siRNA duplex were also shown to abolish RNAi activity. In addition, these studies also indicate that the position of the cleavage site in

the target RNA is defined by the 5'-end of the siRNA guide sequence rather than the 3'-end (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877). Other studies have indicated that a 5'-phosphate on the target-complementary strand of a siRNA duplex is required for siRNA activity and that ATP is utilized to maintain the 5'-phosphate moiety on the siRNA
5 (Nykanen *et al.*, 2001, *Cell*, 107, 309); however, siRNA molecules lacking a 5'-phosphate are active when introduced exogenously, suggesting that 5'-phosphorylation of siRNA constructs may occur *in vivo*.

Synthesis of Nucleic Acid Molecules

Synthesis of nucleic acids greater than 100 nucleotides in length is difficult using
10 automated methods, and the therapeutic cost of such molecules is prohibitive. In this invention, small nucleic acid motifs ("small" refers to nucleic acid motifs no more than 100 nucleotides in length, preferably no more than 80 nucleotides in length, and most preferably no more than 50 nucleotides in length; *e.g.*, individual siNA oligonucleotide sequences or siNA sequences synthesized in tandem) are preferably used for exogenous
15 delivery. The simple structure of these molecules increases the ability of the nucleic acid to invade targeted regions of protein and/or RNA structure. Exemplary molecules of the instant invention are chemically synthesized, and others can similarly be synthesized.

Oligonucleotides (*e.g.*, certain modified oligonucleotides or portions of oligonucleotides lacking ribonucleotides) are synthesized using protocols known in the
20 art, for example as described in Caruthers *et al.*, 1992, *Methods in Enzymology* 211, 3-19, Thompson *et al.*, International PCT Publication No. WO 99/54459, Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677-2684, Wincott *et al.*, 1997, *Methods Mol. Bio.*, 74, 59, Brennan *et al.*, 1998, *Biotechnol Bioeng.*, 61, 33-45, and Brennan, U.S. Pat. No. 6,001,311. All of these references are incorporated herein by reference. The synthesis of
25 oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 μ mol scale protocol with a 2.5 min coupling step for 2'-O-methylated nucleotides and a 45 second coupling step for 2'-deoxy nucleotides or 2'-
30 deoxy-2'-fluoro nucleotides. Table V outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 μ mol scale can be performed on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60

μL of 0.11 M = 6.6 μmol) of 2'-O-methyl phosphoramidite and a 105-fold excess of S-ethyl tetrazole (60 μL of 0.25 M = 15 μmol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 22-fold excess (40 μL of 0.11 M = 4.4 μmol) of deoxy phosphoramidite and a 70-fold excess of S-ethyl tetrazole (40 μL of 0.25 M = 10 μmol) can be used in each coupling cycle of deoxy residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include the following: detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); and oxidation solution is 16.9 mM I₂, 49 mM pyridine, 9% water in THF (PerSeptive Biosystems, Inc.). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide, 0.05 M in acetonitrile) is used.

Deprotection of the DNA-based oligonucleotides is performed as follows: the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aqueous methylamine (1 mL) at 65 °C for 10 minutes. After cooling to -20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H₂O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder.

The method of synthesis used for RNA including certain siNA molecules of the invention follows the procedure as described in Usman *et al.*, 1987, *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.*, 1990, *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677-2684 Wincott *et al.*, 1997, *Methods Mol. Bio.*, 74, 59, and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 μmol scale protocol with a 7.5 min coupling step for alkylsilyl protected nucleotides and a 2.5 min coupling step for 2'-O-methylated nucleotides.

Table V outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 μmol scale can be done on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60 μL of 0.11 M = 6.6 μmol) of 2'-O-methyl phosphoramidite and a 75-fold excess of S-ethyl tetrazole (60 μL of 0.25 M = 15 μmol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 66-fold excess (120 μL of 0.11 M = 13.2 μmol) of alkylsilyl (ribo) protected phosphoramidite and a 150-fold excess of S-ethyl tetrazole (120 μL of 0.25 M = 30 μmol) can be used in each coupling cycle of ribo residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include the following: detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution is 16.9 mM I_2 , 49 mM pyridine, 9% water in THF (PerSeptive Biosystems, Inc.). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide 0.05 M in acetonitrile) is used.

Deprotection of the RNA is performed using either a two-pot or one-pot protocol. For the two-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65 °C for 10 min. After cooling to -20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H₂O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder. The base deprotected oligoribonucleotide is resuspended in anhydrous TEA/HF/NMP solution (300 μL of a solution of 1.5 mL *N*-methylpyrrolidinone, 750 μL TEA and 1 mL TEA•3HF to provide a 1.4 M HF concentration) and heated to 65 °C. After 1.5 h, the oligomer is quenched with 1.5 M NH_4HCO_3 .

Alternatively, for the one-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 33% ethanolic methylamine/DMSO: 1/1 (0.8 mL) at 65 °C for 15 minutes. The vial is brought to room temperature TEA•3HF (0.1 mL) is added and the vial is
5 heated at 65 °C for 15 minutes. The sample is cooled at -20 °C and then quenched with 1.5 M NH_4HCO_3 .

For purification of the trityl-on oligomers, the quenched NH_4HCO_3 solution is loaded onto a C-18 containing cartridge that had been prewashed with acetonitrile followed by 50 mM TEAA. After washing the loaded cartridge with water, the RNA is
10 detritylated with 0.5% TFA for 13 minutes. The cartridge is then washed again with water, salt exchanged with 1 M NaCl and washed with water again. The oligonucleotide is then eluted with 30% acetonitrile.

The average stepwise coupling yields are typically >98% (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684). Those of ordinary skill in the art will recognize that
15 the scale of synthesis can be adapted to be larger or smaller than the example described above including but not limited to 96-well format.

Alternatively, the nucleic acid molecules of the present invention can be synthesized separately and joined together post-synthetically, for example, by ligation (Moore *et al.*, 1992, *Science* 256, 9923; Draper *et al.*, International PCT publication No.
20 WO 93/23569; Shabarova *et al.*, 1991, *Nucleic Acids Research* 19, 4247; Bellon *et al.*, 1997, *Nucleosides & Nucleotides*, 16, 951; Bellon *et al.*, 1997, *Bioconjugate Chem.* 8, 204), or by hybridization following synthesis and/or deprotection.

The siNA molecules of the invention can also be synthesized via a tandem synthesis methodology as described in Example 1 herein, wherein both siNA strands are
25 synthesized as a single contiguous oligonucleotide fragment or strand separated by a cleavable linker which is subsequently cleaved to provide separate siNA fragments or strands that hybridize and permit purification of the siNA duplex. The linker can be a polynucleotide linker or a non-nucleotide linker. The tandem synthesis of siNA as described herein can be readily adapted to both multiwell/multiplate synthesis platforms
30 such as 96 well or similarly larger multi-well platforms. The tandem synthesis of siNA as described herein can also be readily adapted to large scale synthesis platforms employing batch reactors, synthesis columns and the like.

A siNA molecule can also be assembled from two distinct nucleic acid strands or fragments wherein one fragment includes the sense region and the second fragment includes the antisense region of the RNA molecule.

5 The nucleic acid molecules of the present invention can be modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, 2'-O-methyl, 2'-H (for a review see Usman and Cedergren, 1992, *TIBS* 17, 34; Usman *et al.*, 1994, *Nucleic Acids Symp. Ser.* 31, 163). siNA constructs can be purified by gel electrophoresis using general methods or can be purified by high pressure liquid chromatography (HPLC; see Wincott *et al.*, *supra*, the totality of which is
10 hereby incorporated herein by reference) and re-suspended in water.

In another aspect of the invention, siNA molecules of the invention are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. siNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus.
15 The recombinant vectors capable of expressing the siNA molecules can be delivered as described herein, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of siNA molecules.

Optimizing Activity of the nucleic acid molecule of the invention.

Chemically synthesizing nucleic acid molecules with modifications (base, sugar
20 and/or phosphate) can prevent their degradation by serum ribonucleases, which can increase their potency (see *e.g.*, Eckstein *et al.*, International Publication No. WO 92/07065; Perrault *et al.*, 1990 *Nature* 344, 565; Pieken *et al.*, 1991, *Science* 253, 314; Usman and Cedergren, 1992, *Trends in Biochem. Sci.* 17, 334; Usman *et al.*, International Publication No. WO 93/15187; and Rossi *et al.*, International Publication
25 No. WO 91/03162; Sproat, U.S. Pat. No. 5,334,711; Gold *et al.*, U.S. Pat. No. 6,300,074; and Burgin *et al.*, *supra*; all of which are incorporated by reference herein). All of the above references describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of the nucleic acid molecules described herein. Modifications that enhance their efficacy in cells, and removal of bases from nucleic acid
30 molecules to shorten oligonucleotide synthesis times and reduce chemical requirements are desired.

There are several examples in the art describing sugar, base and phosphate modifications that can be introduced into nucleic acid molecules with significant enhancement in their nuclease stability and efficacy. For example, oligonucleotides are modified to enhance stability and/or enhance biological activity by modification with

5 nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, 2'-O-methyl, 2'-O-allyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992, *TIBS*, 17, 34; Usman *et al.*, 1994, *Nucleic Acids Symp. Ser.* 31, 163; Burgin *et al.*, 1996, *Biochemistry*, 35, 14090). Sugar modification of nucleic acid molecules have been extensively described in the art (see Eckstein *et al.*, *International Publication* PCT No.

10 WO 92/07065; Perrault *et al.* *Nature*, 1990, 344, 565-568; Pieken *et al.* *Science*, 1991, 253, 314-317; Usman and Cedergren, *Trends in Biochem. Sci.*, 1992, 17, 334-339; Usman *et al.* *International Publication* PCT No. WO 93/15187; Sproat, *U.S. Pat.* No. 5,334,711 and Beigelman *et al.*, 1995, *J. Biol. Chem.*, 270, 25702; Beigelman *et al.*, *International PCT publication* No. WO 97/26270; Beigelman *et al.*, *U.S. Pat.* No.

15 5,716,824; Usman *et al.*, *U.S. Pat.* No. 5,627,053; Woolf *et al.*, *International PCT Publication* No. WO 98/13526; Thompson *et al.*, *USSN* 60/082,404 which was filed on April 20, 1998; Karpeisky *et al.*, 1998, *Tetrahedron Lett.*, 39, 1131; Earnshaw and Gait, 1998, *Biopolymers (Nucleic Acid Sciences)*, 48, 39-55; Verma and Eckstein, 1998, *Annu. Rev. Biochem.*, 67, 99-134; and Burlina *et al.*, 1997, *Bioorg. Med. Chem.*, 5, 1999-2010;

20 all of the references are hereby incorporated in their totality by reference herein). Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into nucleic acid molecules without modulating catalysis, and are incorporated by reference herein. In view of such teachings, similar modifications can be used as described herein to modify

25 the siNA nucleic acid molecules of the instant invention so long as the ability of siNA to promote RNAi in cells is not significantly inhibited.

While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorodithioate, and/or 5'-methylphosphonate linkages improves stability, excessive modifications can cause some toxicity or decreased activity.

30 Therefore, when designing nucleic acid molecules, the amount of these internucleotide linkages should be minimized. The reduction in the concentration of these linkages should lower toxicity, resulting in increased efficacy and higher specificity of these molecules.

Short interfering nucleic acid (siNA) molecules having chemical modifications that maintain or enhance activity are provided. Such a nucleic acid is also generally more resistant to nucleases than an unmodified nucleic acid. Accordingly, the *in vitro* and/or *in vivo* activity should not be significantly lowered. In cases in which modulation is the goal, therapeutic nucleic acid molecules delivered exogenously should optimally be stable within cells until translation of the target RNA has been modulated long enough to reduce the levels of the undesirable protein. This period of time varies between hours to days depending upon the disease state. Improvements in the chemical synthesis of RNA and DNA (Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677; Caruthers *et al.*, 1992, *Methods in Enzymology* 211, 3-19 (incorporated by reference herein)) have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability, as described above.

In one embodiment, nucleic acid molecules of the invention include one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) G-clamp nucleotides. A G-clamp nucleotide is a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine within a duplex, see for example Lin and Matteucci, 1998, *J. Am. Chem. Soc.*, 120, 8531-8532. A single G-clamp analog substitution within an oligonucleotide can result in substantially enhanced helical thermal stability and mismatch discrimination when hybridized to complementary oligonucleotides. The inclusion of such nucleotides in nucleic acid molecules of the invention results in both enhanced affinity and specificity to nucleic acid targets, complementary sequences, or template strands. In another embodiment, nucleic acid molecules of the invention include one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) LNA "locked nucleic acid" nucleotides such as a 2', 4'-C methylene bicyclo nucleotide (see for example Wengel *et al.*, International PCT Publication No. WO 00/66604 and WO 99/14226).

In another embodiment, the invention features conjugates and/or complexes of siNA molecules of the invention. Such conjugates and/or complexes can be used to facilitate delivery of siNA molecules into a biological system, such as a cell. The conjugates and complexes provided by the instant invention can impart therapeutic activity by transferring therapeutic compounds across cellular membranes, altering the pharmacokinetics, and/or modulating the localization of nucleic acid molecules of the invention. The present invention encompasses the design and synthesis of novel

conjugates and complexes for the delivery of molecules, including, but not limited to, small molecules, lipids, cholesterol, phospholipids, nucleosides, nucleotides, nucleic acids, antibodies, toxins, negatively charged polymers and other polymers, for example proteins, peptides, hormones, carbohydrates, polyethylene glycols, or polyamines, across
5 cellular membranes. In general, the transporters described are designed to be used either individually or as part of a multi-component system, with or without degradable linkers. These compounds are expected to improve delivery and/or localization of nucleic acid molecules of the invention into a number of cell types originating from different tissues, in the presence or absence of serum (see Sullenger and Cech, U.S. Pat. No. 5,854,038).
10 Conjugates of the molecules described herein can be attached to biologically active molecules via linkers that are biodegradable, such as biodegradable nucleic acid linker molecules.

The term "biodegradable linker" as used herein, refers to a nucleic acid or non-nucleic acid linker molecule that is designed as a biodegradable linker to connect one
15 molecule to another molecule, for example, a biologically active molecule to a siNA molecule of the invention or the sense and antisense strands of a siNA molecule of the invention. The biodegradable linker is designed such that its stability can be modulated for a particular purpose, such as delivery to a particular tissue or cell type. The stability of a nucleic acid-based biodegradable linker molecule can be modulated by using various
20 chemistries, for example combinations of ribonucleotides, deoxyribonucleotides, and chemically-modified nucleotides, such as 2'-O-methyl, 2'-fluoro, 2'-amino, 2'-O-amino, 2'-C-allyl, 2'-O-allyl, and other 2'-modified or base modified nucleotides. The biodegradable nucleic acid linker molecule can be a dimer, trimer, tetramer or longer nucleic acid molecule, for example, an oligonucleotide of about 2, 3, 4, 5, 6, 7, 8, 9, 10,
25 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length, or can comprise a single nucleotide with a phosphorus-based linkage, for example, a phosphoramidate or phosphodiester linkage. The biodegradable nucleic acid linker molecule can also comprise nucleic acid backbone, nucleic acid sugar, or nucleic acid base modifications.

The term "biodegradable" as used herein, refers to degradation in a biological
30 system, for example, enzymatic degradation or chemical degradation.

The term "biologically active molecule" as used herein refers to compounds or molecules that are capable of eliciting or modifying a biological response in a system.

Non-limiting examples of biologically active siNA molecules either alone or in combination with other molecules contemplated by the instant invention include therapeutically active molecules such as antibodies, cholesterol, hormones, antivirals, peptides, proteins, chemotherapeutics, small molecules, vitamins, co-factors, nucleosides, nucleotides, oligonucleotides, enzymatic nucleic acids, antisense nucleic acids, triplex forming oligonucleotides, 2,5-A chimeras, siNA, dsRNA, allozymes, aptamers, decoys and analogs thereof. Biologically active molecules of the invention also include molecules capable of modulating the pharmacokinetics and/or pharmacodynamics of other biologically active molecules, for example, lipids and polymers such as polyamines, polyamides, polyethylene glycol and other polyethers.

The term "phospholipid" as used herein, refers to a hydrophobic molecule comprising at least one phosphorus group. For example, a phospholipid can comprise a phosphorus-containing group and saturated or unsaturated alkyl group, optionally substituted with OH, COOH, oxo, amine, or substituted or unsubstituted aryl groups.

Therapeutic nucleic acid molecules (e.g., siNA molecules) delivered exogenously optimally are stable within cells until reverse transcription of the RNA has been modulated long enough to reduce the levels of the RNA transcript. The nucleic acid molecules are resistant to nucleases in order to function as effective intracellular therapeutic agents. Improvements in the chemical synthesis of nucleic acid molecules described in the instant invention and in the art have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability as described above.

In yet another embodiment, siNA molecules having chemical modifications that maintain or enhance enzymatic activity of proteins involved in RNAi are provided. Such nucleic acids are also generally more resistant to nucleases than unmodified nucleic acids. Thus, *in vitro* and/or *in vivo* the activity should not be significantly lowered.

Use of the nucleic acid-based molecules of the invention will lead to better treatments by affording the possibility of combination therapies (e.g., multiple siNA molecules targeted to different genes; nucleic acid molecules coupled with known small molecule modulators; or intermittent treatment with combinations of molecules, including different motifs and/or other chemical or biological molecules). The treatment of subjects with siNA molecules can also include combinations of different types of

nucleic acid molecules, such as enzymatic nucleic acid molecules (ribozymes), allozymes, antisense, 2,5-A oligoadenylate, decoys, and aptamers.

In another aspect a siNA molecule of the invention comprises one or more 5' and/or a 3'-cap structure, for example, on only the sense siNA strand, the antisense siNA strand, or both siNA strands.

By "cap structure" is meant chemical modifications, which have been incorporated at either terminus of the oligonucleotide (see, for example, Adamic *et al.*, U.S. Pat. No. 5,998,203, incorporated by reference herein). These terminal modifications protect the nucleic acid molecule from exonuclease degradation, and may help in delivery and/or localization within a cell. The cap may be present at the 5'-terminus (5'-cap) or at the 3'-terminal (3'-cap) or may be present on both termini. In non-limiting examples, the 5'-cap includes, but is not limited to, glyceryl, inverted deoxy abasic residue (moiety); 4',5'-methylene nucleotide; 1-(beta-D-erythrofuransyl) nucleotide, 4'-thio nucleotide; carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; *threo*-pentofuransyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; or bridging or non-bridging methylphosphonate moiety. Non-limiting examples of cap moieties are shown in Figure 10.

Non-limiting examples of the 3'-cap include, but are not limited to, glyceryl, inverted deoxy abasic residue (moiety), 4', 5'-methylene nucleotide; 1-(beta-D-erythrofuransyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate; 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; *threo*-pentofuransyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moiety; 5'-5'-inverted abasic moiety; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate,

phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties (for more details see Beaucage and Iyer, 1993, *Tetrahedron* 49, 1925; incorporated by reference herein).

By the term "non-nucleotide" is meant any group or compound which can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine and therefore lacks a base at the 1'-position.

An "alkyl" group refers to a saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. Preferably, the alkyl group has 1 to 12 carbons. More preferably, it is a lower alkyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkyl group can be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO₂ or N(CH₃)₂, amino, or SH. The term also includes alkenyl groups that are unsaturated hydrocarbon groups containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has 1 to 12 carbons. More preferably, it is a lower alkenyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkenyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO₂, halogen, N(CH₃)₂, amino, or SH. The term "alkyl" also includes alkynyl groups that have an unsaturated hydrocarbon group containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkynyl group has 1 to 12 carbons. More preferably, it is a lower alkynyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkynyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO₂ or N(CH₃)₂, amino or SH.

Such alkyl groups can also include aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide and ester groups. An "aryl" group refers to an aromatic group that has at least one ring having a conjugated pi electron system and includes carbocyclic aryl, heterocyclic aryl and biaryl groups, all of which may be optionally substituted. The preferred substituent(s) of aryl groups are halogen, trihalomethyl, hydroxyl, SH, OH,

cyano, alkoxy, alkyl, alkenyl, alkynyl, and amino groups. An "alkylaryl" group refers to an alkyl group (as described above) covalently joined to an aryl group (as described above). Carbocyclic aryl groups are groups wherein the ring atoms on the aromatic ring are all carbon atoms. The carbon atoms are optionally substituted. Heterocyclic aryl groups are groups having from 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen, and include furanyl, thienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, imidazolyl and the like, all optionally substituted. An "amide" refers to an -C(O)-NH-R, where R is either alkyl, aryl, alkylaryl or hydrogen. An "ester" refers to an -C(O)-OR', where R is either alkyl, aryl, alkylaryl or hydrogen.

By "nucleotide" as used herein is as recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a nucleotide sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other; see, for example, Usman and McSwiggen, *supra*; Eckstein *et al.*, International PCT Publication No. WO 92/07065; Usman *et al.*, International PCT Publication No. WO 93/15187; Uhlman & Peyman, *supra*, all are hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art as summarized by Limbach *et al.*, 1994, *Nucleic Acids Res.* 22, 2183. Some of the non-limiting examples of base modifications that can be introduced into nucleic acid molecules include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (*e.g.*, 5-methylcytidine), 5-alkyluridines (*e.g.*, ribothymidine), 5-halouridine (*e.g.*, 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (*e.g.* 6-methyluridine), propyne, and others (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090; Uhlman & Peyman, *supra*). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents.

In one embodiment, the invention features modified siNA molecules, with phosphate backbone modifications comprising one or more phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate,

formacetal, thioformacetal, and/or alkylsilyl, substitutions. For a review of oligonucleotide backbone modifications, see Hunziker and Leumann, 1995, *Nucleic Acid Analogues: Synthesis and Properties*, in *Modern Synthetic Methods*, VCH, 331-417, and Mesmaeker *et al.*, 1994, *Novel Backbone Replacements for Oligonucleotides*, in
5 *Carbohydrate Modifications in Antisense Research*, ACS, 24-39.

By "abasic" is meant sugar moieties lacking a base or having other chemical groups in place of a base at the 1' position, see for example Adamic *et al.*, U.S. Pat. No. 5,998,203.

By "unmodified nucleoside" is meant one of the bases adenine, cytosine, guanine,
10 thymine, or uracil joined to the 1' carbon of β -D-ribo-furanose.

By "modified nucleoside" is meant any nucleotide base which contains a modification in the chemical structure of an unmodified nucleotide base, sugar and/or phosphate. Non-limiting examples of modified nucleotides are shown by Formulae I-VII and/or other modifications described herein.

15 In connection with 2'-modified nucleotides as described for the present invention, by "amino" is meant 2'-NH₂ or 2'-O- NH₂, which can be modified or unmodified. Such modified groups are described, for example, in Eckstein *et al.*, U.S. Pat. No. 5,672,695 and Matulic-Adamic *et al.*, U.S. Pat. No. 6,248,878, which are both incorporated by reference in their entireties.

20 Various modifications to nucleic acid siNA structure can be made to enhance the utility of these molecules. Such modifications will enhance shelf-life, half-life *in vitro*, stability, and ease of introduction of such oligonucleotides to the target site, *e.g.*, to enhance penetration of cellular membranes, and confer the ability to recognize and bind to targeted cells.

25 Administration of Nucleic Acid Molecules

A siNA molecule of the invention can be adapted for use to prevent or treat, cancer, proliferative diseases and conditions, and/or ocular diseases and conditions as described herein or otherwise known in the art, or any other trait, disease or condition that is related to or will respond to the levels of HIF1, HIF2, and/or HIF3 in a cell or
30 tissue, alone or in combination with other therapies. For example, a siNA molecule can

comprise a delivery vehicle, including liposomes, for administration to a subject, carriers and diluents and their salts, and/or can be present in pharmaceutically acceptable formulations. Methods for the delivery of nucleic acid molecules are described in Akhtar *et al.*, 1992, *Trends Cell Bio.*, 2, 139; *Delivery Strategies for Antisense Oligonucleotide Therapeutics*, ed. Akhtar, 1995, Maurer *et al.*, 1999, *Mol. Membr. Biol.*, 16, 129-140; Hofland and Huang, 1999, *Handb. Exp. Pharmacol.*, 137, 165-192; and Lee *et al.*, 2000, *ACS Symp. Ser.*, 752, 184-192, all of which are incorporated herein by reference. Beigelman *et al.*, U.S. Pat. No. 6,395,713 and Sullivan *et al.*, PCT WO 94/02595 further describe the general methods for delivery of nucleic acid molecules.

These protocols can be utilized for the delivery of virtually any nucleic acid molecule. Nucleic acid molecules can be administered to cells by a variety of methods known to those of skill in the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as biodegradable polymers, hydrogels, cyclodextrins (see for example Gonzalez *et al.*, 1999, *Bioconjugate Chem.*, 10, 1068-1074; Wang *et al.*, International PCT publication Nos. WO 03/47518 and WO 03/46185), poly(lactic-co-glycolic)acid (PLGA) and PLCA microspheres (see for example US Patent 6,447,796 and US Patent Application Publication No. US 2002130430), biodegradable nanocapsules, and bioadhesive microspheres, or by proteinaceous vectors (O'Hare and Normand, International PCT Publication No. WO 00/53722). In another embodiment, the nucleic acid molecules of the invention can also be formulated or complexed with polyethyleneimine and derivatives thereof, such as polyethyleneimine-polyethyleneglycol-N-acetylgalactosamine (PEI-PEG-GAL) or polyethyleneimine-polyethyleneglycol-tri-N-acetylgalactosamine (PEI-PEG-triGAL) derivatives. In one embodiment, the nucleic acid molecules of the invention are formulated as described in United States Patent Application Publication No. 20030077829, incorporated by reference herein in its entirety.

In one embodiment, a siNA molecule of the invention is complexed with membrane disruptive agents such as those described in U.S. Patent Application Publication No. 20010007666, incorporated by reference herein in its entirety including the drawings. In another embodiment, the membrane disruptive agent or agents and the siNA molecule are also complexed with a cationic lipid or helper lipid molecule, such as those lipids described in U.S. Patent No. 6,235,310, incorporated by reference herein in its entirety including the drawings.

In one embodiment, a siNA molecule of the invention is complexed with delivery systems as described in U.S. Patent Application Publication No. 2003077829 and International PCT Publication Nos. WO 00/03683 and WO 02/087541, all incorporated by reference herein in their entirety including the drawings.

- 5 In one embodiment, delivery systems of the invention include, for example, aqueous and nonaqueous gels, creams, multiple emulsions, microemulsions, liposomes, ointments, aqueous and nonaqueous solutions, lotions, aerosols, hydrocarbon bases and powders, and can contain excipients such as solubilizers, permeation enhancers (e.g., fatty acids, fatty acid esters, fatty alcohols and amino acids), and hydrophilic polymers
- 10 (e.g., polycarboxyl and polyvinylpyrrolidone). In one embodiment, the pharmaceutically acceptable carrier is a liposome or a transdermal enhancer. Examples of liposomes which can be used in this invention include the following: (1) CellFectin, 1:1.5 (M/M) liposome formulation of the cationic lipid N,N,N,N-tetramethyl-N,N,N,N-tetrapalmit-y-spermine and dioleoyl phosphatidylethanolamine (DOPE) (GIBCO BRL); (2) Cytofectin
- 15 GSV, 2:1 (M/M) liposome formulation of a cationic lipid and DOPE (Glen Research); (3) DOTAP (N-[1-(2,3-dioleoyloxy)-N,N,N-tri-methyl-ammoniummethylsulfate] (Boehringer Mannheim); and (4) Lipofectamine, 3:1 (M/M) liposome formulation of the polycationic lipid DOSPA and the neutral lipid DOPE (GIBCO BRL).

- In one embodiment, delivery systems of the invention include patches, tablets,
- 20 suppositories, pessaries, gels and creams, and can contain excipients such as solubilizers and enhancers (e.g., propylene glycol, bile salts and amino acids), and other vehicles (e.g., polyethylene glycol, fatty acid esters and derivatives, and hydrophilic polymers such as hydroxypropylmethylcellulose and hyaluronic acid).

- In one embodiment, siNA molecules of the invention are formulated or complexed
- 25 with polyethylenimine (e.g., linear or branched PEI) and/or polyethylenimine derivatives, including for example grafted PEIs such as galactose PEI, cholesterol PEI, antibody derivatized PEI, and polyethylene glycol PEI (PEG-PEI) derivatives thereof (see for example Ogris *et al.*, 2001, *AAPA PharmSci*, 3, 1-11; Furgeson *et al.*, 2003, *Bioconjugate Chem.*, 14, 840-847; Kunath *et al.*, 2002, *Pharmaceutical Research*, 19, 810-817; Choi *et al.*, 2001, *Bull. Korean Chem. Soc.*, 22, 46-52; Bettinger *et al.*, 1999, *Bioconjugate Chem.*, 10, 558-561; Peterson *et al.*, 2002, *Bioconjugate Chem.*, 13, 845-854; Erbacher *et al.*, 1999, *Journal of Gene Medicine Preprint*, 1, 1-18; Godbey *et al.*,
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1999., PNAS USA, 96, 5177-5181; Godbey et al., 1999, Journal of Controlled Release, 60, 149-160; Diebold et al., 1999, Journal of Biological Chemistry, 274, 19087-19094; Thomas and Klivanov, 2002, PNAS USA, 99, 14640-14645; and Sagara, US 6,586,524, incorporated by reference herein.

5 In one embodiment, a siNA molecule of the invention comprises a bioconjugate, for example a nucleic acid conjugate as described in Vargeese et al., USSN 10/427,160, filed April 30, 2003; US 6,528,631; US 6,335,434; US 6, 235,886; US 6,153,737; US 5,214,136; US 5,138,045, all incorporated by reference herein.

10 Thus, the invention features a pharmaceutical composition comprising one or more nucleic acid(s) of the invention in an acceptable carrier, such as a stabilizer, buffer, and the like. The polynucleotides of the invention can be administered (e.g., RNA, DNA or protein) and introduced to a subject by any standard means, with or without stabilizers, buffers, and the like, to form a pharmaceutical composition. When it is desired to use a liposome delivery mechanism, standard protocols for formation of liposomes can be
15 followed. The compositions of the present invention can also be formulated and used as creams, gels, sprays, oils and other suitable compositions for topical, dermal, or transdermal administration as is known in the art.

 The present invention also includes pharmaceutically acceptable formulations of the compounds described. These formulations include salts of the above compounds,
20 e.g., acid addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid.

 A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, e.g., systemic or local administration, into a cell or subject, including for example a human. Suitable forms, in part, depend
25 upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation from reaching a target cell (i.e., a cell to which the negatively charged nucleic acid is desirable for delivery). For example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms
30 that prevent the composition or formulation from exerting its effect.

In one embodiment, siNA molecules of the invention are administered to a subject by systemic administration in a pharmaceutically acceptable composition or formulation. By "systemic administration" is meant *in vivo* systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body.

5 Administration routes that lead to systemic absorption include, without limitation: intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes exposes the siNA molecules of the invention to an accessible diseased tissue. The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size. The use of a

10 liposome or other drug carrier comprising the compounds of the instant invention can potentially localize the drug, for example, in certain tissue types, such as the tissues of the reticular endothelial system (RES). A liposome formulation that can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach can provide enhanced delivery of the drug to target cells by

15 taking advantage of the specificity of macrophage and lymphocyte immune recognition of abnormal cells.

By "pharmaceutically acceptable formulation" or "pharmaceutically acceptable composition" is meant, a composition or formulation that allows for the effective distribution of the nucleic acid molecules of the instant invention in the physical location

20 most suitable for their desired activity. Non-limiting examples of agents suitable for formulation with the nucleic acid molecules of the instant invention include: P-glycoprotein inhibitors (such as Pluronic P85); biodegradable polymers, such as poly (DL-lactide-co-glycolide) microspheres for sustained release delivery (Emerich, DF *et al.*, 1999, *Cell Transplant*, 8, 47-58); and loaded nanoparticles, such as those made of

25 polybutylcyanoacrylate. Other non-limiting examples of delivery strategies for the nucleic acid molecules of the instant invention include material described in Boado *et al.*, 1998, *J. Pharm. Sci.*, 87, 1308-1315; Tyler *et al.*, 1999, *FEBS Lett.*, 421, 280-284; Pardridge *et al.*, 1995, *PNAS USA*, 92, 5592-5596; Boado, 1995, *Adv. Drug Delivery Rev.*, 15, 73-107; Aldrian-Herrada *et al.*, 1998, *Nucleic Acids Res.*, 26, 4910-4916; and

30 Tyler *et al.*, 1999, *PNAS USA*, 96, 7053-7058.

The invention also features the use of the composition comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). These formulations offer a method for

increasing the accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic *et al.* *Chem. Rev.* 1995, 95, 2601-2627; Ishiwata *et al.*, *Chem. Pharm. Bull.* 1995, 43, 1005-1011). Such liposomes have been shown to accumulate selectively in tumors, presumably by extravasation and capture in the neovascularized target tissues (Lasic *et al.*, *Science* 1995, 267, 1275-1276; Oku *et al.*, 1995, *Biochim. Biophys. Acta*, 1238, 86-90). The long-circulating liposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes which are known to accumulate in tissues of the MPS (Liu *et al.*, *J. Biol. Chem.* 1995, 270, 24864-24870; Choi *et al.*, International PCT Publication No. WO 96/10391; Ansell *et al.*, International PCT Publication No. WO 96/10390; Holland *et al.*, International PCT Publication No. WO 96/10392). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen.

The present invention also includes compositions prepared for storage or administration that include a pharmaceutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co. (A.R. Gennaro edit. 1985), hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents can be provided. These include sodium benzoate, sorbic acid and esters of *p*-hydroxybenzoic acid. In addition, antioxidants and suspending agents can be used.

A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state. The pharmaceutically effective dose depends on the type of disease, the composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors that those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered dependent upon potency of the negatively charged polymer.

The nucleic acid molecules of the invention and formulations thereof can be administered orally, topically, parenterally, by inhalation or spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and/or vehicles. The term parenteral as used herein includes
5 percutaneous, subcutaneous, intravascular (e.g., intravenous), intramuscular, or intrathecal injection or infusion techniques and the like. In addition, there is provided a pharmaceutical formulation comprising a nucleic acid molecule of the invention and a pharmaceutically acceptable carrier. One or more nucleic acid molecules of the invention can be present in association with one or more non-toxic pharmaceutically
10 acceptable carriers and/or diluents and/or adjuvants, and if desired other active ingredients. The pharmaceutical compositions containing nucleic acid molecules of the invention can be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs.

15 Compositions intended for oral use can be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions can contain one or more such sweetening agents, flavoring agents, coloring agents or preservative agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with non-toxic
20 pharmaceutically acceptable excipients that are suitable for the manufacture of tablets. These excipients can be, for example, inert diluents; such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, corn starch, or alginic acid; binding agents, for example starch, gelatin or acacia; and lubricating agents, for example magnesium
25 stearate, stearic acid or talc. The tablets can be uncoated or they can be coated by known techniques. In some cases such coatings can be prepared by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate can be employed.

30 Formulations for oral use can also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example peanut oil, liquid paraffin or olive oil.

Aqueous suspensions contain the active materials in a mixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydropropyl-methylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents can be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions can also contain one or more preservatives, for example ethyl, or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose or saccharin.

Oily suspensions can be formulated by suspending the active ingredients in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions can contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents and flavoring agents can be added to provide palatable oral preparations. These compositions can be preserved by the addition of an anti-oxidant such as ascorbic acid

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents or suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavoring and coloring agents, can also be present.

Pharmaceutical compositions of the invention can also be in the form of oil-in-water emulsions. The oily phase can be a vegetable oil or a mineral oil or mixtures of these. Suitable emulsifying agents can be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally-occurring phosphatides, for example soy bean, lecithin, and esters or partial esters derived from fatty acids and hexitol, anhydrides, for

example sorbitan monooleate, and condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monooleate. The emulsions can also contain sweetening and flavoring agents.

Syrups and elixirs can be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol, glucose or sucrose. Such formulations can also contain a demulcent, a preservative and flavoring and coloring agents. The pharmaceutical compositions can be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension can be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents that have been mentioned above. The sterile injectable preparation can also be a sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

The nucleic acid molecules of the invention can also be administered in the form of suppositories, *e.g.*, for rectal administration of the drug. These compositions can be prepared by mixing the drug with a suitable non-irritating excipient that is solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Such materials include cocoa butter and polyethylene glycols.

Nucleic acid molecules of the invention can be administered parenterally in a sterile medium. The drug, depending on the vehicle and concentration used, can either be suspended or dissolved in the vehicle. Advantageously, adjuvants such as local anesthetics, preservatives and buffering agents can be dissolved in the vehicle.

Dosage levels of the order of from about 0.1 mg to about 140 mg per kilogram of body weight per day are useful in the treatment of the above-indicated conditions (about 0.5 mg to about 7 g per subject per day). The amount of active ingredient that can be combined with the carrier materials to produce a single dosage form varies depending upon the host treated and the particular mode of administration. Dosage unit forms generally contain between from about 1 mg to about 500 mg of an active ingredient.

It is understood that the specific dose level for any particular subject depends upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, and rate of excretion, drug combination and the severity of the particular disease
5 undergoing therapy.

For administration to non-human animals, the composition can also be added to the animal feed or drinking water. It can be convenient to formulate the animal feed and drinking water compositions so that the animal takes in a therapeutically appropriate quantity of the composition along with its diet. It can also be convenient to present the
10 composition as a premix for addition to the feed or drinking water.

The nucleic acid molecules of the present invention can also be administered to a subject in combination with other therapeutic compounds to increase the overall therapeutic effect. The use of multiple compounds to treat an indication can increase the beneficial effects while reducing the presence of side effects.

15 In one embodiment, the invention comprises compositions suitable for administering nucleic acid molecules of the invention to specific cell types. For example, the asialoglycoprotein receptor (ASGPr) (Wu and Wu, 1987, *J. Biol. Chem.* 262, 4429-4432) is unique to hepatocytes and binds branched galactose-terminal glycoproteins, such as asialoorosomucoid (ASOR). In another example, the folate
20 receptor is overexpressed in many cancer cells. Binding of such glycoproteins, synthetic glycoconjugates, or folates to the receptor takes place with an affinity that strongly depends on the degree of branching of the oligosaccharide chain, for example, triantennary structures are bound with greater affinity than biantennary or monoantennary chains (Baenziger and Fiete, 1980, *Cell*, 22, 611-620; Connolly *et al.*, 1982, *J. Biol.*
25 *Chem.*, 257, 939-945). Lee and Lee, 1987, *Glycoconjugate J.*, 4, 317-328, obtained this high specificity through the use of N-acetyl-D-galactosamine as the carbohydrate moiety, which has higher affinity for the receptor, compared to galactose. This "clustering effect" has also been described for the binding and uptake of mannosyl-terminating glycoproteins or glycoconjugates (Ponpipom *et al.*, 1981, *J. Med. Chem.*, 24, 1388-
30 1395). The use of galactose, galactosamine, or folate based conjugates to transport exogenous compounds across cell membranes can provide a targeted delivery approach to, for example, the treatment of liver disease, cancers of the liver, or other cancers. The

use of bioconjugates can also provide a reduction in the required dose of therapeutic compounds required for treatment. Furthermore, therapeutic bioavailability, pharmacodynamics, and pharmacokinetic parameters can be modulated through the use of nucleic acid bioconjugates of the invention. Non-limiting examples of such
5 bioconjugates are described in Vargeese *et al.*, USSN 10/201,394, filed August 13, 2001; and Mahulic-Adamic *et al.*, USSN 60/362,016, filed March 6, 2002.

Alternatively, certain siNA molecules of the instant invention can be expressed within cells from eukaryotic promoters (e.g., Izant and Weintraub, 1985, *Science*, 229, 345; McGarry and Lindquist, 1986, *Proc. Natl. Acad. Sci.*, USA 83, 399; Scanlon *et al.*,
10 1991, *Proc. Natl. Acad. Sci. USA*, 88, 10591-5; Kashani-Sabet *et al.*, 1992, *Antisense Res. Dev.*, 2, 3-15; Dropulic *et al.*, 1992, *J. Virol.*, 66, 1432-41; Weerasinghe *et al.*, 1991, *J. Virol.*, 65, 5531-4; Ojwang *et al.*, 1992, *Proc. Natl. Acad. Sci. USA*, 89, 10802-6; Chen *et al.*, 1992, *Nucleic Acids Res.*, 20, 4581-9; Sarver *et al.*, 1990 *Science*, 247, 1222-1225; Thompson *et al.*, 1995, *Nucleic Acids Res.*, 23, 2259; Good *et al.*, 1997,
15 *Gene Therapy*, 4, 45. Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary transcript by a enzymatic nucleic acid (Draper *et al.*, PCT WO 93/23569, and Sullivan *et al.*, PCT WO 94/02595; Ohkawa *et al.*, 1992, *Nucleic Acids Symp. Ser.*, 27, 15-6; Taira *et al.*, 1991,
20 *Nucleic Acids Res.*, 19, 5125-30; Ventura *et al.*, 1993, *Nucleic Acids Res.*, 21, 3249-55; Chowrira *et al.*, 1994, *J. Biol. Chem.*, 269, 25856.

In another aspect of the invention, RNA molecules of the present invention can be expressed from transcription units (see for example Couture *et al.*, 1996, *TIG.*, 12, 510) inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or
25 viral vectors. siNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. In another embodiment, pol III based constructs are used to express nucleic acid molecules of the invention (see for example Thompson, U.S. Pats. Nos. 5,902,880 and 6,146,886). The recombinant vectors capable of expressing the siNA molecules can be delivered as described above,
30 and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of nucleic acid molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the siNA molecule interacts with the target mRNA and generates an RNAi response. Delivery of siNA molecule expressing vectors

can be systemic, such as by intravenous or intra-muscular administration, by administration to target cells ex-planted from a subject followed by reintroduction into the subject, or by any other means that would allow for introduction into the desired target cell (for a review see Couture *et al.*, 1996, *TIG.*, 12, 510).

5 In one aspect the invention features an expression vector comprising a nucleic acid sequence encoding at least one siNA molecule of the instant invention. The expression vector can encode one or both strands of a siNA duplex, or a single self-complementary strand that self hybridizes into a siNA duplex. The nucleic acid sequences encoding the siNA molecules of the instant invention can be operably linked in a manner that allows
10 expression of the siNA molecule (see for example Paul *et al.*, 2002, *Nature Biotechnology*, 19, 505; Miyagishi and Taira, 2002, *Nature Biotechnology*, 19, 497; Lee *et al.*, 2002, *Nature Biotechnology*, 19, 500; and Novina *et al.*, 2002, *Nature Medicine*, advance online publication doi:10.1038/nm725).

In another aspect, the invention features an expression vector comprising: a) a
15 transcription initiation region (*e.g.*, eukaryotic pol I, II or III initiation region); b) a transcription termination region (*e.g.*, eukaryotic pol I, II or III termination region); and c) a nucleic acid sequence encoding at least one of the siNA molecules of the instant invention, wherein said sequence is operably linked to said initiation region and said termination region in a manner that allows expression and/or delivery of the siNA
20 molecule. The vector can optionally include an open reading frame (ORF) for a protein operably linked on the 5' side or the 3'-side of the sequence encoding the siNA of the invention; and/or an intron (intervening sequences).

Transcription of the siNA molecule sequences can be driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase
25 III (pol III). Transcripts from pol II or pol III promoters are expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type depends on the nature of the gene regulatory sequences (enhancers, silencers, etc.) present nearby. Prokaryotic RNA polymerase promoters are also used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990,
30 *Proc. Natl. Acad. Sci. U S A*, 87, 6743-7; Gao and Huang 1993, *Nucleic Acids Res.*, 21, 2867-72; Lieber *et al.*, 1993, *Methods Enzymol.*, 217, 47-66; Zhou *et al.*, 1990, *Mol. Cell. Biol.*, 10, 4529-37). Several investigators have demonstrated that nucleic acid

molecules expressed from such promoters can function in mammalian cells (e.g. Kashani-Sabet *et al.*, 1992, *Antisense Res. Dev.*, 2, 3-15; Ojwang *et al.*, 1992, *Proc. Natl. Acad. Sci. U S A*, 89, 10802-6; Chen *et al.*, 1992, *Nucleic Acids Res.*, 20, 4581-9; Yu *et al.*, 1993, *Proc. Natl. Acad. Sci. U S A*, 90, 6340-4; L'Huillier *et al.*, 1992, *EMBO J.*, 11, 4411-8; Lisiewicz *et al.*, 1993, *Proc. Natl. Acad. Sci. U. S. A*, 90, 8000-4; Thompson *et al.*, 1995, *Nucleic Acids Res.*, 23, 2259; Sullenger & Cech, 1993, *Science*, 262, 1566). More specifically, transcription units such as the ones derived from genes encoding U6 small nuclear (snRNA), transfer RNA (tRNA) and adenovirus VA RNA are useful in generating high concentrations of desired RNA molecules such as siNA in cells

10 (Thompson *et al.*, *supra*; Couture and Stinchcomb, 1996, *supra*; Noonberg *et al.*, 1994, *Nucleic Acid Res.*, 22, 2830; Noonberg *et al.*, U.S. Pat. No. 5,624,803; Good *et al.*, 1997, *Gene Ther.*, 4, 45; Beigelman *et al.*, International PCT Publication No. WO 96/18736. The above siNA transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors,

15 viral DNA vectors (such as adenovirus or adeno-associated virus vectors), or viral RNA vectors (such as retroviral or alphavirus vectors) (for a review see Couture and Stinchcomb, 1996, *supra*).

In another aspect the invention features an expression vector comprising a nucleic acid sequence encoding at least one of the siNA molecules of the invention in a manner

20 that allows expression of that siNA molecule. The expression vector comprises in one embodiment; a) a transcription initiation region; b) a transcription termination region; and c) a nucleic acid sequence encoding at least one strand of the siNA molecule, wherein the sequence is operably linked to the initiation region and the termination region in a manner that allows expression and/or delivery of the siNA molecule.

25 In another embodiment the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an open reading frame; and d) a nucleic acid sequence encoding at least one strand of a siNA molecule, wherein the sequence is operably linked to the 3'-end of the open reading frame and wherein the sequence is operably linked to the initiation region, the open reading frame and the

30 termination region in a manner that allows expression and/or delivery of the siNA molecule. In yet another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; and d) a nucleic acid sequence encoding at least one siNA molecule, wherein the sequence is

operably linked to the initiation region, the intron and the termination region in a manner which allows expression and/or delivery of the nucleic acid molecule.

In another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; d) an open reading frame; and e) a nucleic acid sequence encoding at least one strand of a siNA molecule, wherein the sequence is operably linked to the 3'-end of the open reading frame and wherein the sequence is operably linked to the initiation region, the intron, the open reading frame and the termination region in a manner which allows expression and/or delivery of the siNA molecule.

10 HIF1 biology and biochemistry

Hypoxia is the result of deprivation of oxygen which can reduce the growth and viability of cells. However, hypoxia also causes an increase in the number of blood vessels and oxygen-carrying capacity of the blood. Hypoxia-inducible factor HIF1 α plays a role in the cellular responses to hypoxia and helps to restore oxygen homeostasis by inducing glycolysis, erythropoiesis and angiogenesis (Carmeliet *et al.*, 1998, *Nature*, 394, 485). HIF1 and VEGF are also believed to play a role in tumor progression by promoting vascularization of tumors. HIF1 is a transcription factor that regulates production of messenger RNA for hypoxia-inducible factors, such as VEGF (Kaelin, 1999, *Nature*, 399, 203). The α subunits of HIF are degraded by proteasome under normal conditions, but are stabilized by hypoxia (Maxwell *et al.*, 1999, *Nature*, 399, 271). When HIF1 is not degraded the result is uncontrolled proliferation of blood vessels. Therefore, tumor vascularization is controlled by the level of HIF1 and to some extent by HIF1's regulation of VEGF (Kamura *et al.*, *Proc. Natl. Acad. Sci. USA*, 97, 10430). The loss of HIF1 can reduce the hypoxia induced expression of vascular endothelial growth factor, prevent formation of large vessels in embryonic stem cell derived tumors, and can impair vascular function which causes a hypoxic microenvironment within the tumor mass (Carmeliet *et al.*, 1998, *Nature*, 394, 485).

There exists the need for therapeutics effective in reversing the physiological changes associated with the maintenance and/or development of angiogenesis, cancer, proliferative diseases, and any other disease or indication that responds to the level of a HIF1 gene in a cell or tissue. Compounds, such as small nucleic acid molecules (e.g., short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded

RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of mediating RNA interference (RNAi), that modulate expression of HIF1, (i.e. to decrease the expression of HIF1), can be used as such a therapeutic.

Examples:

- 5 The following are non-limiting examples showing the selection, isolation, synthesis and activity of nucleic acids of the instant invention.

Example 1: Tandem synthesis of siNA constructs

Exemplary siNA molecules of the invention are synthesized in tandem using a cleavable linker, for example, a succinyl-based linker. Tandem synthesis as described
10 herein is followed by a one-step purification process that provides RNAi molecules in high yield. This approach is highly amenable to siNA synthesis in support of high throughput RNAi screening, and can be readily adapted to multi-column or multi-well synthesis platforms.

After completing a tandem synthesis of a siNA oligo and its complement in which
15 the 5'-terminal dimethoxytrityl (5'-O-DMT) group remains intact (trityl on synthesis), the oligonucleotides are deprotected as described above. Following deprotection, the siNA sequence strands are allowed to spontaneously hybridize. This hybridization yields a duplex in which one strand has retained the 5'-O-DMT group while the complementary strand comprises a terminal 5'-hydroxyl. The newly formed duplex behaves as a single
20 molecule during routine solid-phase extraction purification (Trityl-On purification) even though only one molecule has a dimethoxytrityl group. Because the strands form a stable duplex, this dimethoxytrityl group (or an equivalent group, such as other trityl groups or other hydrophobic moieties) is all that is required to purify the pair of oligos, for example, by using a C18 cartridge.

25 Standard phosphoramidite synthesis chemistry is used up to the point of introducing a tandem linker, such as an inverted deoxy abasic succinate or glyceryl succinate linker (see Figure 1) or an equivalent cleavable linker. A non-limiting example of linker coupling conditions that can be used includes a hindered base such as diisopropylethylamine (DIPA) and/or DMAP in the presence of an activator reagent such
30 as Bromotripyrrolidinophosphoniumhexafluorophosphate (PyBrOP). After the linker is coupled, standard synthesis chemistry is utilized to complete synthesis of the second

sequence leaving the terminal the 5'-O-DMT intact. Following synthesis, the resulting oligonucleotide is deprotected according to the procedures described herein and quenched with a suitable buffer, for example with 50mM NaOAc or 1.5M $\text{NH}_4\text{H}_2\text{CO}_3$.

Purification of the siNA duplex can be readily accomplished using solid phase
5 extraction, for example, using a Waters C18 SepPak 1g cartridge conditioned with 1 column volume (CV) of acetonitrile, 2 CV H_2O , and 2 CV 50mM NaOAc. The sample is loaded and then washed with 1 CV H_2O or 50mM NaOAc. Failure sequences are eluted with 1 CV 14% ACN (Aqueous with 50mM NaOAc and 50mM NaCl). The column is then washed, for example with 1 CV H_2O followed by on-column
10 detritylation, for example by passing 1 CV of 1% aqueous trifluoroacetic acid (TFA) over the column, then adding a second CV of 1% aqueous TFA to the column and allowing to stand for approximately 10 minutes. The remaining TFA solution is removed and the column washed with H_2O followed by 1 CV 1M NaCl and additional H_2O . The siNA duplex product is then eluted, for example, using 1 CV 20% aqueous
15 CAN.

Figure 2 provides an example of MALDI-TOF mass spectrometry analysis of a purified siNA construct in which each peak corresponds to the calculated mass of an individual siNA strand of the siNA duplex. The same purified siNA provides three peaks when analyzed by capillary gel electrophoresis (CGE), one peak presumably
20 corresponding to the duplex siNA, and two peaks presumably corresponding to the separate siNA sequence strands. Ion exchange HPLC analysis of the same siNA construct only shows a single peak. Testing of the purified siNA construct using a luciferase reporter assay described below demonstrated the same RNAi activity compared to siNA constructs generated from separately synthesized oligonucleotide sequence strands.

25 Example 2: Identification of potential siNA target sites in any RNA sequence

The sequence of an RNA target of interest, such as a viral or human mRNA transcript, is screened for target sites, for example by using a computer folding algorithm. In a non-limiting example, the sequence of a gene or RNA gene transcript derived from a database, such as Genbank, is used to generate siNA targets having
30 complementarity to the target. Such sequences can be obtained from a database, or can be determined experimentally as known in the art. Target sites that are known, for example, those target sites determined to be effective target sites based on studies with

other nucleic acid molecules, for example ribozymes or antisense, or those targets known to be associated with a disease or condition such as those sites containing mutations or deletions, can be used to design siNA molecules targeting those sites. Various parameters can be used to determine which sites are the most suitable target sites within the target RNA sequence. These parameters include but are not limited to secondary or tertiary RNA structure, the nucleotide base composition of the target sequence, the degree of homology between various regions of the target sequence, or the relative position of the target sequence within the RNA transcript. Based on these determinations, any number of target sites within the RNA transcript can be chosen to screen siNA molecules for efficacy, for example by using *in vitro* RNA cleavage assays, cell culture, or animal models. In a non-limiting example, anywhere from 1 to 1000 target sites are chosen within the transcript based on the size of the siNA construct to be used. High throughput screening assays can be developed for screening siNA molecules using methods known in the art, such as with multi-well or multi-plate assays to determine efficient reduction in target gene expression.

Example 3: Selection of siNA molecule target sites in a RNA

The following non-limiting steps can be used to carry out the selection of siNAs targeting a given gene sequence or transcript.

1. The target sequence is parsed *in silico* into a list of all fragments or subsequences of a particular length, for example 23 nucleotide fragments, contained within the target sequence. This step is typically carried out using a custom Perl script, but commercial sequence analysis programs such as Oligo, MacVector, or the GCG Wisconsin Package can be employed as well.
2. In some instances the siNAs correspond to more than one target sequence; such would be the case for example in targeting different transcripts of the same gene, targeting different transcripts of more than one gene, or for targeting both the human gene and an animal homolog. In this case, a subsequence list of a particular length is generated for each of the targets, and then the lists are compared to find matching sequences in each list. The subsequences are then ranked according to the number of target sequences that contain the given subsequence; the goal is to find subsequences that are present in most or all of the target sequences. Alternately, the ranking can identify subsequences that are unique to a target sequence, such as a mutant target

sequence. Such an approach would enable the use of siNA to target specifically the mutant sequence and not effect the expression of the normal sequence.

3. In some instances the siNA subsequences are absent in one or more sequences while present in the desired target sequence; such would be the case if the siNA targets a gene with a paralogous family member that is to remain untargeted. As in case 2 above, a subsequence list of a particular length is generated for each of the targets, and then the lists are compared to find sequences that are present in the target gene but are absent in the untargeted paralog.
4. The ranked siNA subsequences can be further analyzed and ranked according to GC content. A preference can be given to sites containing 30-70% GC, with a further preference to sites containing 40-60% GC.
5. The ranked siNA subsequences can be further analyzed and ranked according to self-folding and internal hairpins. Weaker internal folds are preferred; strong hairpin structures are to be avoided.
6. The ranked siNA subsequences can be further analyzed and ranked according to whether they have runs of GGG or CCC in the sequence. GGG (or even more Gs) in either strand can make oligonucleotide synthesis problematic and can potentially interfere with RNAi activity, so it is avoided whenever better sequences are available. CCC is searched in the target strand because that will place GGG in the antisense strand.
7. The ranked siNA subsequences can be further analyzed and ranked according to whether they have the dinucleotide UU (uridine dinucleotide) on the 3'-end of the sequence, and/or AA on the 5'-end of the sequence (to yield 3' UU on the antisense sequence). These sequences allow one to design siNA molecules with terminal TT thymidine dinucleotides.
8. Four or five target sites are chosen from the ranked list of subsequences as described above. For example, in subsequences having 23 nucleotides, the right 21 nucleotides of each chosen 23-mer subsequence are then designed and synthesized for the upper (sense) strand of the siNA duplex, while the reverse complement of the left 21 nucleotides of each chosen 23-mer subsequence are then designed and synthesized for the lower (antisense) strand of the siNA duplex (see Tables II and III). If terminal

TT residues are desired for the sequence (as described in paragraph 7), then the two 3' terminal nucleotides of both the sense and antisense strands are replaced by TT prior to synthesizing the oligos.

9. The siNA molecules are screened in an *in vitro*, cell culture or animal model system to identify the most active siNA molecule or the most preferred target site within the target RNA sequence.
10. Other design considerations can be used when selecting target nucleic acid sequences, see, for example, Reynolds *et al.*, 2004, *Nature Biotechnology Advanced Online Publication*, 1 February 2004, doi:10.1038/nbt936 and Ui-Tei *et al.*, 2004, *Nucleic Acids Research*, 32, doi:10.1093/nar/gkh247.

In an alternate approach, a pool of siNA constructs specific to a HIF1 target sequence is used to screen for target sites in cells expressing HIF1 RNA, such as cultured such as human T cells. The general strategy used in this approach is shown in Figure 9. A non-limiting example of such is a pool comprising sequences having any of SEQ ID NOS 1-562. Cells expressing HIF1 (e.g., Hepa-1 cells, such as Hepa-1c1c7, Hepa-1c4, Hepa-1c31, and Hepa-1Rc4 cells) are transfected with the pool of siNA constructs and cells that demonstrate a phenotype associated with HIF1 inhibition are sorted. The pool of siNA constructs can be expressed from transcription cassettes inserted into appropriate vectors (see for example Figure 7 and Figure 8). The siNA from cells demonstrating a positive phenotypic change (e.g., decreased proliferation, decreased HIF1 mRNA levels or decreased HIF1 protein expression), are sequenced to determine the most suitable target site(s) within the target HIF1 RNA sequence.

Example 4: HIF1 targeted siNA design

siNA target sites were chosen by analyzing sequences of the HIF1 RNA target and optionally prioritizing the target sites on the basis of folding (structure of any given sequence analyzed to determine siNA accessibility to the target), by using a library of siNA molecules as described in Example 3, or alternately by using an *in vitro* siNA system as described in Example 6 herein. siNA molecules were designed that could bind each target and are optionally individually analyzed by computer folding to assess whether the siNA molecule can interact with the target sequence. Varying the length of the siNA molecules can be chosen to optimize activity. Generally, a sufficient number

of complementary nucleotide bases are chosen to bind to, or otherwise interact with, the target RNA, but the degree of complementarity can be modulated to accommodate siNA duplexes or varying length or base composition. By using such methodologies, siNA molecules can be designed to target sites within any known RNA sequence, for example
5 those RNA sequences corresponding to the any gene transcript.

Chemically modified siNA constructs are designed to provide nuclease stability for systemic administration in vivo and/or improved pharmacokinetic, localization, and delivery properties while preserving the ability to mediate RNAi activity. Chemical modifications as described herein are introduced synthetically using synthetic methods
10 described herein and those generally known in the art. The synthetic siNA constructs are then assayed for nuclease stability in serum and/or cellular/tissue extracts (e.g. liver extracts). The synthetic siNA constructs are also tested in parallel for RNAi activity using an appropriate assay, such as a luciferase reporter assay as described herein or another suitable assay that can quantify RNAi activity. Synthetic siNA constructs that
15 possess both nuclease stability and RNAi activity can be further modified and re-evaluated in stability and activity assays. The chemical modifications of the stabilized active siNA constructs can then be applied to any siNA sequence targeting any chosen RNA and used, for example, in target screening assays to pick lead siNA compounds for therapeutic development (see for example Figure 11).

20 Example 5: Chemical Synthesis and Purification of siNA

siNA molecules can be designed to interact with various sites in the RNA message, for example, target sequences within the RNA sequences described herein. The sequence of one strand of the siNA molecule(s) is complementary to the target site sequences described above. The siNA molecules can be chemically synthesized using
25 methods described herein. Inactive siNA molecules that are used as control sequences can be synthesized by scrambling the sequence of the siNA molecules such that it is not complementary to the target sequence. Generally, siNA constructs can be synthesized using solid phase oligonucleotide synthesis methods as described herein (see for example Usman *et al.*, US Patent Nos. 5,804,683; 5,831,071; 5,998,203; 6,117,657; 6,353,098;
30 6,362,323; 6,437,117; 6,469,158; Scaringe *et al.*, US Patent Nos. 6,111,086; 6,008,400; 6,111,086 all incorporated by reference herein in their entirety).

In a non-limiting example, RNA oligonucleotides are synthesized in a stepwise fashion using the phosphoramidite chemistry as is known in the art. Standard phosphoramidite chemistry involves the use of nucleosides comprising any of 5'-O-dimethoxytrityl, 2'-O-tert-butyldimethylsilyl, 3'-O-2-Cyanoethyl N,N-diisopropylphosphoroamidite groups, and exocyclic amine protecting groups (e.g. N6-benzoyl adenosine, N4 acetyl cytidine, and N2-isobutyryl guanosine). Alternately, 2'-O-Silyl Ethers can be used in conjunction with acid-labile 2'-O-orthoester protecting groups in the synthesis of RNA as described by Scaringe *supra*. Differing 2' chemistries can require different protecting groups, for example 2'-deoxy-2'-amino nucleosides can utilize N-phthaloyl protection as described by Usman *et al.*, US Patent 5,631,360, incorporated by reference herein in its entirety).

During solid phase synthesis, each nucleotide is added sequentially (3'- to 5'-direction) to the solid support-bound oligonucleotide. The first nucleoside at the 3'-end of the chain is covalently attached to a solid support (e.g., controlled pore glass or polystyrene) using various linkers. The nucleotide precursor, a ribonucleoside phosphoramidite, and activator are combined resulting in the coupling of the second nucleoside phosphoramidite onto the 5'-end of the first nucleoside. The support is then washed and any unreacted 5'-hydroxyl groups are capped with a capping reagent such as acetic anhydride to yield inactive 5'-acetyl moieties. The trivalent phosphorus linkage is then oxidized to a more stable phosphate linkage. At the end of the nucleotide addition cycle, the 5'-O-protecting group is cleaved under suitable conditions (e.g., acidic conditions for trityl-based groups and Fluoride for silyl-based groups). The cycle is repeated for each subsequent nucleotide.

Modification of synthesis conditions can be used to optimize coupling efficiency, for example by using differing coupling times, differing reagent/phosphoramidite concentrations, differing contact times, differing solid supports and solid support linker chemistries depending on the particular chemical composition of the siNA to be synthesized. Deprotection and purification of the siNA can be performed as is generally described in Usman *et al.*, US 5,831,071, US 6,353,098, US 6,437,117, and Bellon *et al.*, US 6,054,576, US 6,162,909, US 6,303,773, or Scaringe *supra*, incorporated by reference herein in their entireties. Additionally, deprotection conditions can be modified to provide the best possible yield and purity of siNA constructs. For example, applicant has observed that oligonucleotides comprising 2'-deoxy-2'-fluoro nucleotides

can degrade under inappropriate deprotection conditions. Such oligonucleotides are deprotected using aqueous methylamine at about 35°C for 30 minutes. If the 2'-deoxy-2'-fluoro containing oligonucleotide also comprises ribonucleotides, after deprotection with aqueous methylamine at about 35°C for 30 minutes, TEA-HF is added and the
5 reaction maintained at about 65°C for an additional 15 minutes.

Example 6: RNAi *in vitro* assay to assess siNA activity

An *in vitro* assay that recapitulates RNAi in a cell-free system is used to evaluate siNA constructs targeting HIF1 RNA targets. The assay comprises the system described by Tuschl *et al.*, 1999, *Genes and Development*, 13, 3191-3197 and Zamore *et al.*, 2000,
10 *Cell*, 101, 25-33 adapted for use with HIF1 target RNA. A *Drosophila* extract derived from syncytial blastoderm is used to reconstitute RNAi activity *in vitro*. Target RNA is generated via *in vitro* transcription from an appropriate HIF1 expressing plasmid using T7 RNA polymerase or via chemical synthesis as described herein. Sense and antisense siNA strands (for example 20 uM each) are annealed by incubation in buffer (such as
15 100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) for 1 minute at 90°C followed by 1 hour at 37°C, then diluted in lysis buffer (for example 100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2mM magnesium acetate). Annealing can be monitored by gel electrophoresis on an agarose gel in TBE buffer and stained with ethidium bromide. The *Drosophila* lysate is prepared using zero to two-
20 hour-old embryos from Oregon R flies collected on yeasted molasses agar that are dechorionated and lysed. The lysate is centrifuged and the supernatant isolated. The assay comprises a reaction mixture containing 50% lysate [vol/vol], RNA (10-50 pM final concentration), and 10% [vol/vol] lysis buffer containing siNA (10 nM final concentration). The reaction mixture also contains 10 mM creatine phosphate, 10 ug/ml
25 creatine phosphokinase, 100 uM GTP, 100 uM UTP, 100 uM CTP, 500 uM ATP, 5 mM DTT, 0.1 U/uL RNasin (Promega), and 100 uM of each amino acid. The final concentration of potassium acetate is adjusted to 100 mM. The reactions are pre-assembled on ice and preincubated at 25° C for 10 minutes before adding RNA, then incubated at 25° C for an additional 60 minutes. Reactions are quenched with 4 volumes
30 of 1.25 x Passive Lysis Buffer (Promega). Target RNA cleavage is assayed by RT-PCR analysis or other methods known in the art and are compared to control reactions in which siNA is omitted from the reaction.

Alternately, internally-labeled target RNA for the assay is prepared by *in vitro* transcription in the presence of [α - 32 P] CTP, passed over a G50 Sephadex column by spin chromatography and used as target RNA without further purification. Optionally, target RNA is 5'- 32 P-end labeled using T4 polynucleotide kinase enzyme. Assays are performed as described above and target RNA and the specific RNA cleavage products generated by RNAi are visualized on an autoradiograph of a gel. The percentage of cleavage is determined by PHOSPHOR IMAGER[®] (autoradiography) quantitation of bands representing intact control RNA or RNA from control reactions without siNA and the cleavage products generated by the assay.

In one embodiment, this assay is used to determine target sites in the HIF1 RNA target for siNA mediated RNAi cleavage, wherein a plurality of siNA constructs are screened for RNAi mediated cleavage of the HIF1 RNA target, for example, by analyzing the assay reaction by electrophoresis of labeled target RNA, or by northern blotting, as well as by other methodology well known in the art.

Example 7: Nucleic acid inhibition of HIF1 target RNA

siNA molecules targeted to the human HIF1 RNA are designed and synthesized as described above. These nucleic acid molecules can be tested for cleavage activity *in vivo*, for example, using the following procedure. The target sequences and the nucleotide location within the HIF1 RNA are given in Tables II and III.

Two formats are used to test the efficacy of siNAs targeting HIF1. First, the reagents are tested in cell culture using, for example, cultured Hepa-1 or A549 cells, to determine the extent of RNA and protein inhibition. siNA reagents (*e.g.*; see Tables II and III) are selected against the HIF1 target as described herein. RNA inhibition is measured after delivery of these reagents by a suitable transfection agent to, for example, Hepa-1 or A549 cells. Relative amounts of target RNA are measured versus actin using real-time PCR monitoring of amplification (*e.g.*, ABI 7700 TAQMAN[®]). A comparison is made to a mixture of oligonucleotide sequences made to unrelated targets or to a randomized siNA control with the same overall length and chemistry, but randomly substituted at each position. Primary and secondary lead reagents are chosen for the target and optimization performed. After an optimal transfection agent concentration is

chosen, a RNA time-course of inhibition is performed with the lead siNA molecule. In addition, a cell-plating format can be used to determine RNA inhibition.

Delivery of siNA to Cells

Cells (e.g., cultured Hepa-1 or A549 cells) are seeded, for example, at 1×10^5 cells per well of a six-well dish in EGM-2 (BioWhittaker) the day before transfection. siNA (final concentration, for example 20nM) and cationic lipid (e.g., final concentration 2 μ g/ml) are complexed in EGM basal media (Bio Whittaker) at 37°C for 30 minutes in polystyrene tubes. Following vortexing, the complexed siNA is added to each well and incubated for the times indicated. For initial optimization experiments, cells are seeded, for example, at 1×10^3 in 96 well plates and siNA complex added as described. Efficiency of delivery of siNA to cells is determined using a fluorescent siNA complexed with lipid. Cells in 6-well dishes are incubated with siNA for 24 hours, rinsed with PBS and fixed in 2% paraformaldehyde for 15 minutes at room temperature. Uptake of siNA is visualized using a fluorescent microscope.

TAQMAN® (real-time PCR monitoring of amplification) and Lightcycler quantification of mRNA

Total RNA is prepared from cells following siNA delivery, for example, using Qiagen RNA purification kits for 6-well or Rneasy extraction kits for 96-well assays. For TAQMAN® analysis (real-time PCR monitoring of amplification), dual-labeled probes are synthesized with the reporter dye, FAM or JOE, covalently linked at the 5'-end and the quencher dye TAMRA conjugated to the 3'-end. One-step RT-PCR amplifications are performed on, for example, an ABI PRISM 7700 Sequence Detector using 50 μ l reactions consisting of 10 μ l total RNA, 100 nM forward primer, 900 nM reverse primer, 100 nM probe, 1X TaqMan PCR reaction buffer (PE-Applied Biosystems), 5.5 mM MgCl₂, 300 μ M each dATP, dCTP, dGTP, and dTTP, 10U RNase Inhibitor (Promega), 1.25U AMPLITAQ GOLD® (DNA polymerase) (PE-Applied Biosystems) and 10U M-MLV Reverse Transcriptase (Promega). The thermal cycling conditions can consist of 30 minutes at 48°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Quantitation of mRNA levels is determined relative to standards generated from serially diluted total cellular RNA (300, 100, 33, 11 ng/rxn) and normalizing to β -actin or GAPDH mRNA in parallel TAQMAN® reactions (real-time

- PCR monitoring of amplification). For each gene of interest an upper and lower primer and a fluorescently labeled probe are designed. Real time incorporation of SYBR Green I dye into a specific PCR product can be measured in glass capillary tubes using a lightcycler. A standard curve is generated for each primer pair using control cRNA.
- 5 Values are represented as relative expression to GAPDH in each sample.

Western blotting

- Nuclear extracts can be prepared using a standard micro preparation technique (see for example Andrews and Faller, 1991, *Nucleic Acids Research*, 19, 2499). Protein extracts from supernatants are prepared, for example using TCA precipitation. An equal
- 10 volume of 20% TCA is added to the cell supernatant, incubated on ice for 1 hour and pelleted by centrifugation for 5 minutes. Pellets are washed in acetone, dried and resuspended in water. Cellular protein extracts are run on a 10% Bis-Tris NuPage (nuclear extracts) or 4-12% Tris-Glycine (supernatant extracts) polyacrylamide gel and transferred onto nitro-cellulose membranes. Non-specific binding can be blocked by
- 15 incubation, for example, with 5% non-fat milk for 1 hour followed by primary antibody for 16 hour at 4°C. Following washes, the secondary antibody is applied, for example (1:10,000 dilution) for 1 hour at room temperature and the signal detected with SuperSignal reagent (Pierce).

Example 8: Models useful to evaluate the down-regulation of HIF1 gene expression

20 *Cell Culture*

- There are numerous cell culture systems that can be used to analyze reduction of HIF1 levels either directly or indirectly by measuring downstream effects. For example, Hepa-1 or A549 cells can be used in cell culture experiments to assess the efficacy of nucleic acid molecules of the invention. As such, cells treated with nucleic acid
- 25 molecules of the invention (e.g., siNA) targeting HIF1 RNA would be expected to have decreased HIF1 expression capacity compared to matched control nucleic acid molecules having a scrambled or inactive sequence. In a non-limiting example, Hepa-1 or A549 cells are cultured and HIF1 expression is quantified, for example by time-resolved immuno fluorometric assay. HIF1 messenger-RNA expression is quantitated with RT-
- 30 PCR in cultured cells. Untreated cells are compared to cells treated with siNA molecules transfected with a suitable reagent, for example a cationic lipid such as lipofectamine,

and HIF1 protein and RNA levels are quantitated. Dose response assays are then performed to establish dose dependent inhibition of HIF1 expression. In a non-limiting example, cell culture experiments are adapted to those experiments described in Carmeliet *et al.*, 1998, *Nature*, 394, 485 or Maxwell *et al.*, 1997, *PNAS USA*, 94, 8104).

- 5 In several cell culture systems, cationic lipids have been shown to enhance the bioavailability of oligonucleotides to cells in culture (Bennet, *et al.*, 1992, *Mol. Pharmacology*, 41, 1023-1033). In one embodiment, siNA molecules of the invention are complexed with cationic lipids for cell culture experiments. siNA and cationic lipid mixtures are prepared in serum-free DMEM immediately prior to addition to the cells.
- 10 DMEM plus additives are warmed to room temperature (about 20-25°C) and cationic lipid is added to the final desired concentration and the solution is vortexed briefly. siNA molecules are added to the final desired concentration and the solution is again vortexed briefly and incubated for 10 minutes at room temperature. In dose response experiments, the RNA/lipid complex is serially diluted into DMEM following the 10
- 15 minute incubation.

Animal Models

- Evaluating the efficacy of anti-HIF1 agents in animal models is an important prerequisite to human clinical trials. The role of HIF1 has recently been investigated (Maxwell *et al.*, 1997, *Proc. Natl. Acad. Sci. USA*, 94, 8104) using Hepa-1 cells and
- 20 selected derivatives as xenografts in immunodeficient mice, to assess the effect of HIF1 on tumor behavior since solid tumors contain regions of hypoxia. Investigators have evaluated the activation of HIF1 in Hepa-1 cells that are wild type, defective (c4), and revertant (Rc4) for HIF1 β /ARNT. Given that HIF1 systems activated by hypoxia contain elevated glucose transporter (GLUT3), it can be used as a marker for HIF1
- 25 activation. Results suggested that HIF1 was activated in regions of the tumors (Maxwell *et al.*, *supra*). Investigators also examined the effect of HIF1 on VEGF. Results indicated that there was increased expression of VEGF around necrotic regions of the tumors and that hypoxia is responsible for the effect (Maxwell *et al.*, *supra*). In addition, vascularization of c4 tumors was reduced compared with wild type and Rc4 tumors,
- 30 suggesting that hypoxia, acting via HIF1 dependent changes, is an important factor in the angiogenic process (Maxwell *et al.*, *supra*). The quantitative differences in

vascularization were not confined to restricted regions but to the distance to the nearest capillary.

The animal model described by Maxwell *et al.*, *supra*, can be used to evaluate inhibition of HIF1 expression, as well as changes in the vascularization and development of angiogenic characteristics in the presence of an HIF1 blockade, such as an siNA molecule of the invention. For example, the improved clearance of tumors in this mouse model can be associated with HIF1 blockade resulting from administration of an siNA molecule of the invention. These results indicate that manipulation of HIF1 can be used toward therapeutic use in preventing and/or treating cancer and other proliferative conditions discussed herein in mammalian subjects, including humans.

Example 9: RNAi mediated inhibition of HIF1 expression

siNA constructs (Table III) are tested for efficacy in reducing HIF1 RNA expression in, for example, Hepa-1 or A549 cells. Cells are plated approximately 24 hours before transfection in 96-well plates at 5,000-7,500 cells/well, 100 µl/well, such that at the time of transfection cells are 70-90% confluent. For transfection, annealed siNAs are mixed with the transfection reagent (Lipofectamine 2000, Invitrogen) in a volume of 50 µl/well and incubated for 20 minutes at room temperature. The siNA transfection mixtures are added to cells to give a final siNA concentration of 25 nM in a volume of 150 µl. Each siNA transfection mixture is added to 3 wells for triplicate siNA treatments. Cells are incubated at 37° for 24 hours in the continued presence of the siNA transfection mixture. At 24 hours, RNA is prepared from each well of treated cells. The supernatants with the transfection mixtures are first removed and discarded, then the cells are lysed and RNA prepared from each well. Target gene expression following treatment is evaluated by RT-PCR for the target gene and for a control gene (36B4, an RNA polymerase subunit) for normalization. The triplicate data is averaged and the standard deviations determined for each treatment. Normalized data are graphed and the percent reduction of target mRNA by active siNAs in comparison to their respective inverted control siNAs is determined.

In a non-limiting example, chemically modified siNA constructs (Table III) were tested for efficacy as described above in reducing HIF1 RNA expression in A549 cells. Active siNAs were evaluated compared to untreated cells, a matched chemistry irrelevant control (IC), and a transfection control. Results are summarized in Figure 22. Figure

22 shows results for chemically modified siNA constructs targeting various sites in HIF1 mRNA. As shown in Figure 22, the active siNA constructs provide significant inhibition of HIF1 gene expression in cell culture experiments as determined by levels of HIF1 mRNA when compared to appropriate controls.

5 Example 10: Indications

The present body of knowledge in inhibitors of apoptosis research indicates the need for methods and compounds that can regulate HIF1 gene expression for research, diagnostic, and therapeutic use. As described herein, the nucleic acid molecules of the present invention can be used to treat or prevent cancer, proliferative diseases and
10 conditions, ocular diseases and conditions, and any other indications that can respond to the level of hypoxia inducible factors such as HIF1 in a cell or tissue as described herein or otherwise known in the art.

The use of radiation treatments and chemotherapeutics, such as Gemcytabine and cyclophosphamide, are non-limiting examples of chemotherapeutic agents that can be
15 combined with or used in conjunction with the nucleic acid molecules (e.g. siNA molecules) of the instant invention. Those skilled in the art will recognize that other anti-cancer compounds and therapies can similarly be readily combined with the nucleic acid molecules of the instant invention (e.g. siNA molecules) and are hence within the scope of the instant invention. Such compounds and therapies are well known in the art
20 (see for example *Cancer: Principles and Practice of Oncology*, Volumes 1 and 2, eds Devita, V.T., Hellman, S., and Rosenberg, S.A., J.B. Lippincott Company, Philadelphia, USA; incorporated herein by reference) and include, without limitation, folates, antifolates, pyrimidine analogs, fluoropyrimidines, purine analogs, adenosine analogs, topoisomerase I inhibitors, anthracyclins, platinum
25 analogs, alkylating agents, nitrosoureas, plant derived compounds such as vinca alkaloids, epipodophyllotoxins, tyrosine kinase inhibitors, taxols, radiation therapy, surgery, nutritional supplements, gene therapy, radiotherapy, for example 3D-CRT, immunotoxin therapy, for example ricin, and monoclonal antibodies. Specific examples
30 of chemotherapeutic compounds that can be combined with or used in conjunction with the nucleic acid molecules of the invention include, but are not limited to, Paclitaxel; Docetaxel; Methotrexate; Doxorubin; Edoxate; Vinorelbine; Tomaxifen; Leucovorin; 5-fluoro uridine (5-FU); Isonitrocan; Cisplatin; Carboplatin; Amsacrine; Cytarabine;

Bleomycin; Mitomycin C; Dactinomycin; Mithramycin; Hexamethylmelamine; Dacarbazine; L-asparaginase; Nitrogen mustard; Melphalan, Chlorambucil; Busulfan; Ifosfamide; 4-hydroperoxycyclophosphamide; Thiotepa; Irinotecan (CAMPTOSAR®, CPT-11, Camptothecin-11, Campto) Tamoxifen; Herceptin; IMC C225; ABX-EGF; and combinations thereof. The above list of compounds are non-limiting examples of compounds and/or methods that can be combined with or used in conjunction with the nucleic acid molecules (e.g. siNA) of the instant invention. Those skilled in the art will recognize that other drug compounds and therapies can similarly be readily combined with the nucleic acid molecules of the instant invention (e.g., siNA molecules) are hence within the scope of the instant invention.

Example 11: Diagnostic uses

The siNA molecules of the invention can be used in a variety of diagnostic applications, such as in the identification of molecular targets (e.g., RNA) in a variety of applications, for example, in clinical, industrial, environmental, agricultural and/or research settings. Such diagnostic use of siNA molecules involves utilizing reconstituted RNAi systems, for example, using cellular lysates or partially purified cellular lysates. siNA molecules of this invention can be used as diagnostic tools to examine genetic drift and mutations within diseased cells or to detect the presence of endogenous or exogenous, for example viral, RNA in a cell. The close relationship between siNA activity and the structure of the target RNA allows the detection of mutations in any region of the molecule, which alters the base-pairing and three-dimensional structure of the target RNA. By using multiple siNA molecules described in this invention, one can map nucleotide changes, which are important to RNA structure and function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with siNA molecules can be used to inhibit gene expression and define the role of specified gene products in the progression of disease or infection. In this manner, other genetic targets can be defined as important mediators of the disease. These experiments will lead to better treatment of the disease progression by affording the possibility of combination therapies (e.g., multiple siNA molecules targeted to different genes, siNA molecules coupled with known small molecule inhibitors, or intermittent treatment with combinations siNA molecules and/or other chemical or biological molecules). Other *in vitro* uses of siNA molecules of this invention are well known in the art, and include detection of the presence of mRNAs associated with a disease, infection, or related condition. Such

RNA is detected by determining the presence of a cleavage product after treatment with a siNA using standard methodologies, for example, fluorescence resonance emission transfer (FRET).

5 In a specific example, siNA molecules that cleave only wild-type or mutant forms of the target RNA are used for the assay. The first siNA molecules (*i.e.*, those that cleave only wild-type forms of target RNA) are used to identify wild-type RNA present in the sample and the second siNA molecules (*i.e.*, those that cleave only mutant forms of target RNA) are used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of both wild-type and mutant RNA are cleaved by both siNA
10 molecules to demonstrate the relative siNA efficiencies in the reactions and the absence of cleavage of the "non-targeted" RNA species. The cleavage products from the synthetic substrates also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population. Thus, each analysis requires two siNA molecules, two substrates and one unknown sample, which is combined into six
15 reactions. The presence of cleavage products is determined using an RNase protection assay so that full-length and cleavage fragments of each RNA can be analyzed in one lane of a polyacrylamide gel. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired phenotypic changes in target cells. The expression of mRNA whose protein product is implicated in
20 the development of the phenotype (*i.e.*, disease related or infection related) is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels is adequate and decreases the cost of the initial diagnosis. Higher mutant form to wild-type ratios are correlated with higher risk whether RNA levels are compared qualitatively or quantitatively.

25 All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. All references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

30 One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods and compositions described herein as presently representative of preferred embodiments are exemplary and are not intended as

limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art, which are encompassed within the spirit of the invention, are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and
5 modifications can be made to the invention disclosed herein without departing from the scope and spirit of the invention. Thus, such additional embodiments are within the scope of the present invention and the following claims. The present invention teaches one skilled in the art to test various combinations and/or substitutions of chemical
10 modifications described herein toward generating nucleic acid constructs with improved activity for mediating RNAi activity. Such improved activity can comprise improved stability, improved bioavailability, and/or improved activation of cellular responses mediating RNAi. Therefore, the specific embodiments described herein are not limiting and one skilled in the art can readily appreciate that specific combinations of the
15 modifications described herein can be tested without undue experimentation toward identifying siNA molecules with improved RNAi activity.

The invention illustratively described herein suitably can be practiced in the absence of any element or elements, limitation or limitations that are not specifically disclosed herein. Thus, for example, in each instance herein any of the terms
20 "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although
25 the present invention has been specifically disclosed by preferred embodiments, optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the description and the appended claims.

In addition, where features or aspects of the invention are described in terms of
30 Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

Table I: HIF1 Accession Numbers

- 5 LOCUS NM_001530 3958 bp mRNA linear PRI 27-MAY-2003
 DEFINITION Homo sapiens hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor) (HIF1A), transcript variant 1, mRNA.
 ACCESSION NM_001530
- 10 HIFA HUMAN
 * Size: 826 amino acids; 92670 Da
 * Function: INVOLVED IN THE INDUCTION OF OXYGEN REGULATED GENES. SPECIFICALLY RECOGNIZES AN 8 BP HYPOXIA RESPONSE ELEMENT (HRE).
- 15 * Subunit: EFFICIENT DNA BINDING REQUIRES DIMERIZATION WITH ANOTHER BHLH PROTEIN. HETERODIMER OF AN ALPHA AND A BETA (ARNT) SUBUNITS. THE ALPHA SUBUNIT IS UNIQUE TO HIF-1 WHEREAS HIF-1 BETA (ARNT) CAN DIMERIZE WITH OTHER BHLH-PAS PROTEINS. INTERACTS WITH HSP90.
- 20 * Subcellular location: Nuclear (Potential).
 * Induction: UNDER REDUCED OXYGEN TENSION.
 * Domain: BOTH HYPOXIA RESPONSIVENESS AND TRANSACTIVATION CAPABILITY RESIDE WITHIN THE C-TERMINAL PART.
 * Ptm: REQUIRES PHOSPHORYLATION FOR DNA-BINDING.
- 25 * Similarity: BELONGS TO THE BASIC HELIX-LOOP-HELIX (BHLH) FAMILY OF TRANSCRIPTION FACTORS.
 * Similarity: Contains 2 PAS (PER-ARNT-SIM) dimerization domains.
 * Similarity: Contains 1 PAS-associated C-terminal (PAC) domain.
-
- 30 LOCUS NM_001668 4846 bp mRNA linear PRI 16-MAY-2003
 DEFINITION Homo sapiens aryl hydrocarbon receptor nuclear translocator (ARNT), transcript variant 1, mRNA (alias HIFB).
 ACCESSION NM_001668
- 35 ARNT HUMAN
 * Size: 789 amino acids; 86636 Da
 * Function: REQUIRED FOR ACTIVITY OF THE AH (DIOXIN) RECEPTOR. THIS PROTEIN IS REQUIRED FOR THE LIGAND-BINDING SUBUNIT TO TRANSLOCATE FROM THE CYTOSOL TO THE NUCLEUS AFTER
- 40 LIGAND BINDING. THE COMPLEX THEN INITIATES TRANSCRIPTION OF A GENES INVOLVED IN THE ACTIVATION OF PAH PROCARCINOGENS.
- * Subunit: EFFICIENT DNA BINDING REQUIRES DIMERIZATION WITH ANOTHER BHLH PROTEIN. FORMS AN HETERODIMER WITH AHR, WITH HIF1A AS WELL AS WITH OTHER BHLH PROTEINS. INTERACTS WITH TRANSFORMING ACIDIC COILED-COIL CONTAINING PROTEIN 3 (BY SIMILARITY).
- 45 * Subcellular location: Nuclear.
 * Alternative products: Alternative splicing.

- * **Similarity:** BELONGS TO THE BASIC HELIX-LOOP-HELIX (BHLH) FAMILY OF TRANSCRIPTION FACTORS.
 - * **Similarity:** Contains 2 PAS (PER-ARNT-SIM) dimerization domains.
 - * **Similarity:** Contains 1 PAS-associated C-terminal (PAC) domain.
-

5

LOCUS NM_152794 2717 bp mRNA linear PRI 07-MAY-2003
DEFINITION Homo sapiens hypoxia inducible factor 3, alpha subunit (HIF3A), transcript variant 1, mRNA.
ACCESSION NM_152794

10

LOCUS NM_022462 2626 bp mRNA linear PRI 07-MAY-2003
DEFINITION Homo sapiens hypoxia inducible factor 3, alpha subunit (HIF3A), transcript variant 2, mRNA.
ACCESSION NM_022462

15

LOCUS NM_152795 2090 bp mRNA linear PRI 07-MAY-2003
DEFINITION Homo sapiens hypoxia inducible factor 3, alpha subunit (HIF3A), transcript variant 3, mRNA.
ACCESSION NM_152795

20

LOCUS NM_152796 1735 bp mRNA linear PRI 07-MAY-2003
DEFINITION Homo sapiens hypoxia inducible factor 3, alpha subunit (HIF3A), transcript variant 4, mRNA.
ACCESSION NM_152796

25

LOCUS NM_006389 4552 bp mRNA linear PRI 06-APR-2003
DEFINITION Homo sapiens hypoxia up-regulated 1 (HYOU1), mRNA.
ACCESSION NM_006389

30 OXR P HUMAN

- * **Size:** 999 amino acids; 111335 Da
 - * **Function:** HAS A PIVOTAL ROLE IN CYTOPROTECTIVE CELLULAR MECHANISMS TRIGGERED BY OXYGEN DEPRIVATION. MAY PLAY A ROLE AS A MOLECULAR CHAPERONE AND PARTICIPATE IN PROTEIN FOLDING.
 - * **Subcellular location:** Endoplasmic reticulum lumen.
 - * **Induction:** BY HYPOXIA AND ALSO BY 2-DEOXYGLUCOSE OR TUNICAMYCIN.
 - * **Similarity:** BELONGS TO THE HEAT SHOCK PROTEIN 70 FAMILY.
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LOCUS NM_014056 1362 bp mRNA linear PRI 07-MAY-2003
DEFINITION Homo sapiens likely ortholog of mouse hypoxia induced gene 1 (HIG1), mRNA.
ACCESSION NM_014056

45

LOCUS NM_013332 1372 bp mRNA linear PRI 07-APR-2003
DEFINITION Homo sapiens hypoxia-inducible protein 2 (HIG2), mRNA.
ACCESSION NM_013332

50

LOCUS NM_017902 2964 bp mRNA linear PRI 07-APR-2003

DEFINITION Homo sapiens hypoxia-inducible factor 1, alpha subunit inhibitor (HIF1AN), mRNA.

5 ACCESSION NM_017902 NM_032215

TABLE II: HIF1 siNA AND TARGET SEQUENCES

HIF1 NM_001530.2

Pos	Seq	Seq ID	UPos	Upper seq	Seq ID	LPos	Lower seq	Seq ID
3	GCUGCCUCGUCAGGGGA	1	3	GCUGCCUCGUCAGGGGA	1	21	UCCCCUCAGACGAGGCAGC	221
21	ACAGGAGGAUACCCUCU	2	21	ACAGGAGGAUACCCUCU	2	39	AAGAGGGUGAUCCUCUGU	222
39	UCGUCGCUUCGGCCAGUG	3	39	UCGUCGCUUCGGCCAGUG	3	57	ACACUGGCCGAGAGCGAGA	223
57	UGUCGGGCUUGGCCUGAC	4	57	UGUCGGGCUUGGCCUGAC	4	75	GUCAGGGCCAGGCCGACA	224
75	CAAGCCACCUAGGAGAGG	5	75	CAAGCCACCUAGGAGAGG	5	93	CCUCUCUCAGGUGGCUUG	225
93	GCUCGGAGCCGGCCCGGA	6	93	GCUCGGAGCCGGCCCGGA	6	111	UCCGGGCCGCGCUCGAGC	226
111	ACCCGCGCAUUGCCGCC	7	111	ACCCGCGCAUUGCCGCC	7	129	GGCGGCAUUCGCGGGU	227
129	CGCUUCUCUAGUCUCAC	8	129	CGCUUCUCUAGUCUCAC	8	147	GUGAGCAUAGAGAGAGCG	228
147	CGAGGGGUUCCGCCUCG	9	147	CGAGGGGUUCCGCCUCG	8	165	CGAGGCGGAAACCCUCG	229
165	GCACCCCAUCCGCGGAGU	10	165	GCACCCCAUCCGCGGAGU	10	183	AGUCCAGAGGUGGGGUGC	230
183	UUGCCUUCUUCUCUCU	11	183	UUGCCUUCUUCUCUCU	11	201	AGAAAGAGAGGAAAGCAA	231
201	UCCGCGUGGAGGGAGCC	12	201	UCCGCGUGGAGGGAGCC	12	219	GGCUCGCCUCCACACGCGGA	232
219	CAGCGCUAGGCCGAGCG	13	219	CAGCGCUAGGCCGAGCG	13	237	CGCUCGGGCCUAGCGCUG	233
237	GAGCCUGGGGCCGCCCGC	14	237	GAGCCUGGGGCCGCCCGC	14	255	GCGGCGGCGCCCGAGGCU	234
255	CCGUGAAGACAUCCGCGG	15	255	CCGUGAAGACAUCCGCGG	15	273	CCCCGCAUGUCUUCACGG	235
273	GACCGAUACCAUGGAGG	16	273	GACCGAUACCAUGGAGG	16	291	CCUCCAUUGGUAUCCGUG	236
291	GGCGCCGCGCGCGAAG	17	291	GGCGCCGCGCGCGAAG	17	309	CGUCCGCGCGCGCGCGCC	237
309	GACAAGAAAGAUAGUU	18	309	GACAAGAAAGAUAGUU	18	327	AACUUAUCUUUUUCUUGUC	238
327	UCUGAACGUCGAAAGAAA	19	327	UCUGAACGUCGAAAGAAA	19	345	UUUUCUUUCGACGUUCAGA	239
345	AAGUCGAGAUAGCAGCA	20	345	AAGUCGAGAUAGCAGCA	20	363	UGGCUCAUCUCGAGACUU	240
363	AGAUCCGCGAAGUAAAG	21	363	AGAUCCGCGAAGUAAAG	21	381	CUUUACUUCGCCGAGAUUC	241
381	GAAUCGAGUUUUUAUG	22	381	GAAUCGAGUUUUUAUG	22	399	CAUAAAAACUUCAGAUUC	242
399	GAGCUUGCUCAUCAGUUG	23	399	GAGCUUGCUCAUCAGUUG	23	417	GCAACUGAUGAGCAAGCUC	243
417	CCACUCCACAUAAUGUGA	24	417	CCACUCCACAUAAUGUGA	24	435	UCACAUUAUGUGGAAGUGG	244
435	AGUUCGCAUCUUGAUAAAG	25	435	AGUUCGCAUCUUGAUAAAG	25	453	CCUUUAUCAAGAUUGCGAACU	245
453	GCCUCUGAUGAGGCUUA	26	453	GCCUCUGAUGAGGCUUA	26	471	UAAGCCUUCACACAGAGGC	246
471	ACCAUCAGCUAUUUGCGUG	27	471	ACCAUCAGCUAUUUGCGUG	27	489	CACGCAAAUAGCUGAUUGGU	247
489	GUGAGGAAACUUCUGGAUG	28	489	GUGAGGAAACUUCUGGAUG	28	507	CAUCCAGAAUUUCCUCAC	248

507	GCUGGUGAUUUGGAUUAUUG	29	507	GCUGGUGAUUUGGAUUAUUG	29	525	CAAUUCCAAAUUACCCAGC	249
525	GAAGAUGACAUAAAAGCAC	30	525	GAAGAUGACAUAAAAGCAC	30	543	GUGCUUUCAUUAUUAUUC	250
543	CAGAUGAAUUGCUUUUAU	31	543	CAGAUGAAUUGCUUUUAU	31	561	AUAUAAAGCAUUAUUAUUC	251
561	UUGAAAGCCUUGGAUGGUU	32	561	UUGAAAGCCUUGGAUGGUU	32	579	AACCAUCCAAAGGUUUUCAA	252
579	UUUGUUAUUGGUUUCUCACAG	33	579	UUUGUUAUUGGUUUCUCACAG	33	597	CUGUGAAGAACCAUUAACAAA	253
597	GAUGAUGGUGACAUUAU	34	597	GAUGAUGGUGACAUUAU	34	615	AAUUGAGUCCACCAUUAU	254
615	UACAUUUCUGAUAAUUGUGA	35	615	UACAUUUCUGAUAAUUGUGA	35	633	UCACAUUAUUCAGAAUUGUA	255
633	AACAAUUAUUGGGAUUA	36	633	AACAAUUAUUGGGAUUA	36	651	UUAAUCCCAUUAUUAUUGU	256
651	ACUCAGUUUGAACUAACUG	37	651	ACUCAGUUUGAACUAACUG	37	669	CAGUUAUUAUUAUUAUUGU	257
669	GGACACAGUGUGUUUGAUU	38	669	GGACACAGUGUGUUUGAUU	38	687	AAUCAAACACACUGUGUCC	258
687	UUUACUUAUCCAUUGUAC	39	687	UUUACUUAUCCAUUGUAC	39	705	GGUCACAUUGGAUGAGUAAA	259
705	CAUGAGGAAUUGAGAGAAA	40	705	CAUGAGGAAUUGAGAGAAA	40	723	UUUCUCUAUUAUCCUUAUG	260
723	AUGCUUACACACAGAAUUG	41	723	AUGCUUACACACAGAAUUG	41	741	CAUUAUCUGUGUGUAAGCAU	261
741	GGCCUUGUGAAAAAGGUUA	42	741	GGCCUUGUGAAAAAGGUUA	42	759	UACCCUUUUUUCACAAGGCC	262
759	AAAGAACAAAAACACACAGC	43	759	AAAGAACAAAAACACACAGC	43	777	GCUGUGUGUUUUUGUUUUU	263
777	CGAAGCUUUUUUCUCAGAA	44	777	CGAAGCUUUUUUCUCAGAA	44	795	UUUCUGAGAAAAAGCUUCG	264
795	AUGAAGUGUACCCUAACUA	45	795	AUGAAGUGUACCCUAACUA	45	813	UAGUUAAGGGUACACUUAU	265
813	AGCCGAGGAAGAACUAUGA	46	813	AGCCGAGGAAGAACUAUGA	46	831	UCAUAGUUCUUCUCCUGGCU	266
831	AACAUAAAGUCUGCAACAU	47	831	AACAUAAAGUCUGCAACAU	47	849	AUGUUGCAGACUUAUUAUUGU	267
849	UGGAAGGUUAUUGCACUGCA	48	849	UGGAAGGUUAUUGCACUGCA	48	867	UGCAGUGCAAUUACCUUCCA	268
867	ACAGGCCACAUUACAGUAU	49	867	ACAGGCCACAUUACAGUAU	49	885	AUACGUGAAUUGUGGCCUGU	269
885	UAUGAUACCAACAGUAACC	50	885	UAUGAUACCAACAGUAACC	50	903	GGUUAUCUGUUGGUAUUAU	270
903	CAACCUACAGUGUGGUUAUA	51	903	CAACCUACAGUGUGGUUAUA	51	921	UAUACCCACACUGAGGUUG	271
921	AAGAAACCAUUAUAGACCU	52	921	AAGAAACCAUUAUAGACCU	52	939	AGGUCAUAGGUUGGUUUUUCU	272
939	UGCUUUGUGUGAUUUUGUG	53	939	UGCUUUGUGUGAUUUUGUG	53	957	CACAAUACAGCAACCAAGCA	273
957	GAACCCAUUCCUCACCCAU	54	957	GAACCCAUUCCUCACCCAU	54	975	AUGGGUGAGGAUUGGGUUC	274
975	UCAAUUAUUGAAUUAUCCU	55	975	UCAAUUAUUGAAUUAUCCU	55	993	AAGGAUUUUAUUAUUAUUA	275
993	UUAGAUAGCAAGACUUAUCC	56	993	UUAGAUAGCAAGACUUAUCC	56	1011	GGAAAGUCUUGCUUAUUAU	276
1011	CUCAGUCGACACAGCCUGG	57	1011	CUCAGUCGACACAGCCUGG	57	1029	CCAGGCUGUGUGCGACUGAG	277
1029	GAUUGAAUUUUUCUUAU	58	1029	GAUUGAAUUUUUCUUAU	58	1047	AUAUAGAAAUUAUUAUUAU	278
1047	UGUGAUGAAAGAAUUAACCG	59	1047	UGUGAUGAAAGAAUUAACCG	59	1065	CGGUAAUUAUUAUUAUUAU	279
1065	GAUUGAUGGGAUUAUAGC	60	1065	GAUUGAUGGGAUUAUAGC	60	1083	GCUCAUUAUCCCAUUAUUAU	280
1083	CCAGAAGAACUUUAUAGGCC	61	1083	CCAGAAGAACUUUAUAGGCC	61	1101	GGCCUAAAAGUUAUUAUUAU	281
1101	CGCUCAAUUUAUUAUUAUUAU	62	1101	CGCUCAAUUUAUUAUUAUUAU	62	1119	AUAUUAUUAUUAUUAUUAU	282

1119	UAUCAUGCUUUGGACUCUG	63	1118	UAUCAUGCUUUGGACUCUG	63	1137	CAGAGUCCAAAGCAUGAUA	283
1137	GAUCAUCUGACCAAAACUC	64	1137	GAUCAUCUGACCAAAACUC	64	1155	GAGUUUUGGUCAGAUCAUC	284
1155	CAUCAUGAUUUGUUUACUA	65	1155	CAUCAUGAUUUGUUUACUA	65	1173	UAGUAAACAUAUCAUGAUG	285
1173	AAAGGACAAGUCACCACAG	66	1173	AAAGGACAAGUCACCACAG	66	1181	CUGUGGUGACUUGUCCUUU	286
1191	GGACAGUACAGGAGUCUUG	67	1191	GGACAGUACAGGAGUCUUG	67	1209	CAAGCAUCCUGUACUGUCC	287
1209	GCCAAAAGAGGUGGAUAUG	68	1209	GCCAAAAGAGGUGGAUAUG	68	1227	CAUAUCCACCUCUUUUUGGC	288
1227	GUCUGGGUUGAAACUCAAG	69	1227	GUCUGGGUUGAAACUCAAG	69	1245	CUUGAGUUUCAAACCCAGAC	289
1245	GCAACUGUCAUAUAUAACA	70	1245	GCAACUGUCAUAUAUAACA	70	1263	UGUUUAUAUAGACAGUUGC	290
1263	ACCAAGAAUUCUCAAACAC	71	1263	ACCAAGAAUUCUCAAACAC	71	1281	GUGGUUGAGAAUUCUUGGU	291
1281	CAGUGCAUUGUAUGUGUA	72	1281	CAGUGCAUUGUAUGUGUA	72	1299	UCACACUAACAUAUGCACUG	292
1299	AAUACGUUUGAGUGGUA	73	1299	AAUACGUUUGAGUGGUA	73	1317	UACCACUCACAACGUAUU	293
1317	AUUUAUCAGCAGCAGUUA	74	1317	AUUUAUCAGCAGCAGUUA	74	1335	UCAAGUCGUGCUGAAUAAU	294
1335	AUUUUCUCCCUJCAACAAA	75	1335	AUUUUCUCCCUJCAACAAA	75	1353	UUUUGUUGAAGGGAGAAAAU	295
1353	ACAGAAUGUGUCCUUAAC	76	1353	ACAGAAUGUGUCCUUAAC	76	1371	GUUUAAGGACACAUUCUGU	296
1371	CCGGUUGAAUUCUAGAUUA	77	1371	CCGGUUGAAUUCUAGAUUA	77	1389	UAUCUGAAGAUUCAACCCGG	297
1389	AUGAAAUGACUCAGCUAU	78	1389	AUGAAAUGACUCAGCUAU	78	1407	AUAGCUGAGUCAUUUUCAU	298
1407	UUCACCAAGUUGAAUCAG	79	1407	UUCACCAAGUUGAAUCAG	79	1425	CUGAUUCAACUUGGUGAA	299
1425	GAAGAUACAAGUAGCCUCU	80	1425	GAAGAUACAAGUAGCCUCU	80	1443	AGAGGCUACUUGUAUCUUC	300
1443	UUUGACAACUUAAGAAGG	81	1443	UUUGACAACUUAAGAAGG	81	1461	CCUUCUUAAGUUUUGUCAAA	301
1461	GAACCUGAUGCUUUUACUU	82	1461	GAACCUGAUGCUUUUACUU	82	1478	AAGUUAAGCAUCAGGUUC	302
1479	UUUCUGGCCCCAGCCGCUG	83	1479	UUUCUGGCCCCAGCCGCUG	83	1497	CAGCGGCUGGGGCCAGCAA	303
1497	GGAGACACAUAUAUUCUU	84	1497	GGAGACACAUAUAUUCUU	84	1515	AAGAUUAUUGUGUCUCC	304
1515	UUAGAUUUUGGAGCAACG	85	1515	UUAGAUUUUGGAGCAACG	85	1533	CGUUGCUGCCCAAAUCUAA	305
1533	GACACAGAAACUGAUGACC	86	1533	GACACAGAAACUGAUGACC	86	1551	GGUCAUCAGUUUCUGUGUC	306
1551	CAGCAACUUGAGGAAGUAC	87	1551	CAGCAACUUGAGGAAGUAC	87	1569	GUACUCCUCAAGUUGCUG	307
1569	CCAUUAUAUAUUGAUGUAA	88	1569	CCAUUAUAUAUUGAUGUAA	88	1587	UUACAUCAUUAUAUAUUGG	308
1587	AUGCUCCCCUACACCCAACG	89	1587	AUGCUCCCCUACACCCAACG	89	1605	CGUUGGGUGAGGGGAGCAU	309
1605	GAAAAUUACAGAAUAUA	90	1605	GAAAAUUACAGAAUAUA	90	1623	UUUAUUUCUGUAUUUUUC	310
1623	AAUUUGGCAUUGUCUCCAU	91	1623	AAUUUGGCAUUGUCUCCAU	91	1641	AUGGAGACAUUGCCAAAUU	311
1641	UUACCCACCGCUGAAACGC	92	1641	UUACCCACCGCUGAAACGC	92	1659	GCGUUUCAGCGGUGGGUAA	312
1659	CCAAAGCCACUUCGAAGUA	93	1659	CCAAAGCCACUUCGAAGUA	93	1677	UACUUCGAAGUGGCUUUGG	313
1677	AGUGCUGACCCUGCACUCA	94	1677	AGUGCUGACCCUGCACUCA	94	1695	UGAGUGCAGGGUCAGCACU	314
1695	AAUCAAGAAGUUGCAUUA	95	1695	AAUCAAGAAGUUGCAUUA	95	1713	UUAUUGCAACUUCUUGAUU	315
1713	AAAUUAGAACCAAAUCCAG	96	1713	AAAUUAGAACCAAAUCCAG	96	1731	CUGGAUUUGGUUCUUAUUU	316

1731	GAGUCACUGGAACUUCUU	97	1731	GAGUCACUGGAACUUCUU	97	1749	AAGAAAGUUCAGUGACUC	317
1749	UUUACCAUGCCCCAGAUUC	98	1749	UUUACCAUGCCCCAGAUUC	98	1767	GAAUCUGGGGCAUGGJAAA	318
1767	CAGGAUCAGACACCUAGUC	99	1767	CAGGAUCAGACACCUAGUC	99	1785	GACUAGGUGUCUGAUCCUG	319
1785	CCUCCGGAUGGAAGCACUA	100	1785	CCUCCGGAUGGAAGCACUA	100	1803	UAGUGCUUCCAUCCGGAAGG	320
1803	AGACAAAGUUCACCUAGGC	101	1803	AGACAAAGUUCACCUAGGC	101	1821	GCUCAGGUGAACUUCUUGUCU	321
1821	CCUAAUAGUCCCGAGUGAAU	102	1821	CCUAAUAGUCCCGAGUGAAU	102	1839	AUUCACUGGGGACUUAUAGG	322
1839	UAUUGUUUUUAUGUGGAUA	103	1839	UAUUGUUUUUAUGUGGAUA	103	1857	UAUCCACAUAAAAACAUA	323
1857	AGUGAUUUGGUCAUUGAAU	104	1857	AGUGAUUUGGUCAUUGAAU	104	1875	AUUCAUUGACCAUUAUCACU	324
1875	UUCAAGUUUUUGGUGUAG	105	1875	UUCAAGUUUUUGGUGUAG	105	1893	CUACCAUUCACCAUUGAA	325
1893	GAUAAACUUUUUGGUGAAG	106	1893	GAUAAACUUUUUGGUGAAG	106	1911	CUUCAGCAAAAAGUUUUUC	326
1911	GACACAGAAGCAAAAGAAC	107	1911	GACACAGAAGCAAAAGAAC	107	1929	GGUUCUUUGCUUCUGUGUC	327
1929	CCAUUUUUAUAGUUGGACA	108	1929	CCAUUUUUAUAGUUGGACA	108	1947	UGUCCUGAGUAGAAAAUGG	328
1947	ACAGAUUUAAGACUUGGAGA	109	1947	ACAGAUUUAAGACUUGGAGA	109	1965	UCUCCAAAGUCUAAAUCUGU	329
1965	AUGUJAGCUCCCUAUUUC	110	1965	AUGUJAGCUCCCUAUUUC	110	1983	GGAUUAGGGAGCUAACAU	330
1983	CCAUGGAGUAGACUUC	111	1983	CCAUGGAGUAGACUUC	111	2001	GGAAGUCAUCAUCCAUUGG	331
2001	CAGUACGUUCCUUCGUAUC	112	2001	CAGUACGUUCCUUCGUAUC	112	2019	GAUCGAAGGAACGUAACUG	332
2019	CAGUUGUACCAUUAAGAAA	113	2019	CAGUUGUACCAUUAAGAAA	113	2037	UUUCUAUUGGUGACAAACUG	333
2037	AGCAGUCCGCAAGCCUCAA	114	2037	AGCAGUCCGCAAGCCUCAA	114	2055	CAGGCUUUGCGGAACUGCU	334
2055	GAAAGCGCAAGUCCUCAA	115	2055	GAAAGCGCAAGUCCUCAA	115	2073	UUUGAGGACUUGCGCUUUC	335
2073	AGCACAGUUAAGAUUUC	116	2073	AGCACAGUUAAGAUUUC	116	2091	GGAAUACUGUAACUGUGCU	336
2091	CAGCAGACUCAAUAACAAG	117	2091	CAGCAGACUCAAUAACAAG	117	2109	CUUGUAUUUGAGUCUGCUG	337
2109	GAACCUACUGCUAAUGCCA	118	2109	GAACCUACUGCUAAUGCCA	118	2127	UGGCAUUAGCAGUAGGUUC	338
2127	ACCACUACACUGCCACCA	119	2127	ACCACUACACUGCCACCA	119	2145	UGGUGGCAGUGGUAUGGU	339
2145	ACUGAUGAAUUAUAAACAG	120	2145	ACUGAUGAAUUAUAAACAG	120	2163	CUGUUUUUAUUUCAUCAGU	340
2163	GUGACAAAAGACCGUAUGG	121	2163	GUGACAAAAGACCGUAUGG	121	2181	CCAUACGGUCUUUUGUCAC	341
2181	GAAGACAUUAAAUUAUUGA	122	2181	GAAGACAUUAAAUUAUUGA	122	2199	UCAAUUUUUUAUUGUCUUC	342
2199	AUUGCAUCUCCAUUCUCCUA	123	2199	AUUGCAUCUCCAUUCUCCUA	123	2217	UAGGAGUUGGAGAUUGCAAU	343
2217	ACCACAUACAUAAAGAAA	124	2217	ACCACAUACAUAAAGAAA	124	2235	UUUCUUUAUUAUUGUGGU	344
2235	ACUACUAGUGCCACAUCAU	125	2235	ACUACUAGUGCCACAUCAU	125	2253	AUGAUGUGGCACUAGUAGU	345
2253	UCACCAUUAUAGAGUAUCUC	126	2253	UCACCAUUAUAGAGUAUCUC	126	2271	GAGUAUCUCUAUAUUGGUGA	346
2271	CAAGUCGGACAGCCUCAC	127	2271	CAAGUCGGACAGCCUCAC	127	2289	GUGAGGCUUGCCGACUUG	347
2289	CCAAACAGAGCAGGAAAAG	128	2289	CCAAACAGAGCAGGAAAAG	128	2307	CUUUUCCUGCUCUGUUUGG	348
2307	GGAGUCAUAGAACAGACAG	129	2307	GGAGUCAUAGAACAGACAG	129	2325	CUGUCUGUUCUAUGACUCC	349
2325	GAAAAUUCUACUCCAAGAA	130	2325	GAAAAUUCUACUCCAAGAA	130	2343	UUUCUUGGAGAGAUUUUUC	350

2343	AGCCUAACGUGUUAUCUG	131	2343	AGCCUAACGUGUUAUCUG	131	2381	CAGUAACACGUAAGGCU	351
2361	GUCGUUUGAGUCAAAGAA	132	2361	GUCGUUUGAGUCAAAGAA	132	2379	UUCUUUGACUCAAAGCGAC	352
2379	ACUACAGUUCUGAGGAG	133	2379	ACUACAGUUCUGAGGAG	133	2397	CUUCCUCAGGAACUGUAGU	353
2397	GAACUAAUCCAAAGAUAC	134	2397	GAACUAAUCCAAAGAUAC	134	2415	GUACUUIUGGUAUUAUUC	354
2415	CUAGCUUUGCAGAAUGCUC	135	2415	CUAGCUUUGCAGAAUGCUC	135	2433	GAGCAUUCUGCAAAGCUAG	355
2433	CAGAGAAAGCGAAAAUUG	136	2433	CAGAGAAAGCGAAAAUUG	136	2451	CCAUUUUUCGUUUCUCUG	356
2451	GAACAUAGUGGUACAUUU	137	2451	GAACAUAGUGGUACAUUU	137	2469	AAAGUGAACCAUCAUGUUC	357
2469	UUUCAAGCAGUAGGAUUUG	138	2469	UUUCAAGCAGUAGGAUUUG	138	2487	CAAUCCUACUGCUUGAAA	358
2487	GGAACAUUAUACAGCAGC	139	2487	GGAACAUUAUACAGCAGC	139	2505	GCUGCUGUAUAUAUUGUCC	359
2505	CCAGAGCAUCAUGCAGCUA	140	2505	CCAGAGCAUCAUGCAGCUA	140	2523	UAGCUGCAUGAUCGUCUGG	360
2523	ACUACAUACAUUUUCUUGGA	141	2523	ACUACAUACAUUUUCUUGGA	141	2541	UCCAAGAAAGUAUGUAGU	361
2541	AAACGUGUAAAAGGAUGCA	142	2541	AAACGUGUAAAAGGAUGCA	142	2559	UGCAUCCUUUUACACGUAU	362
2559	AAUUCUAGUGAACAGAAUG	143	2559	AAUUCUAGUGAACAGAAUG	143	2577	CAUUCUGUUCACUAGAUUU	363
2577	GGAUUGGAGCAAAAGACAA	144	2577	GGAUUGGAGCAAAAGACAA	144	2595	UUGUCUUUUUCUCCAUUCC	364
2595	AUUUUUUAAUACCCUCUG	145	2595	AUUUUUUAAUACCCUCUG	145	2613	CAGAGGGUAUUAUAAUUAU	365
2613	GAUUUAGCAUGUAGACUGC	146	2613	GAUUUAGCAUGUAGACUGC	146	2631	GCAGUCUACAUUGCUAAUUC	366
2631	CUGGGCAUCAAUGGAUG	147	2631	CUGGGCAUCAAUGGAUG	147	2649	CAUCCAUUGAUUGCCCCCAG	367
2649	GAAUGUGAUUACACAGC	148	2649	GAAUGUGAUUACACAGC	148	2667	GCUGUGGUAUCCACUUAUC	368
2667	CUGACCAGUUAUUGUUG	149	2667	CUGACCAGUUAUUGUUG	149	2685	CACAUCAUAAACUGGUCAG	369
2685	GAAGUUAUUGCUUUAUAC	150	2685	GAAGUUAUUGCUUUAUAC	150	2703	GUUAAGGAGCAUUAACUUC	370
2703	CAAGGCAGCAGAAACCUAC	151	2703	CAAGGCAGCAGAAACCUAC	151	2721	GUAGGUUUUCUGCUGCCUUG	371
2721	CUGCAGGUGAAGAAUUAAC	152	2721	CUGCAGGUGAAGAAUUAAC	152	2739	GUAAUUUCUACCCUGCAG	372
2739	CUCAGAGCUUUGGAUCAAG	153	2739	CUCAGAGCUUUGGAUCAAG	153	2757	CUUGAUCCAAAGCUCUGAG	373
2757	GUUAACUGAGCUUUUUCUU	154	2757	GUUAACUGAGCUUUUUCUU	154	2775	AAGAAAAGGCUAGUUAAC	374
2775	UAAUUUCAUCCUUUUUUU	155	2775	UAAUUUCAUCCUUUUUUU	155	2793	AAAAAAGGAUAGAAUUA	375
2793	UGGACACUGGUGGCUCACU	156	2793	UGGACACUGGUGGCUCACU	156	2811	AGUGAGCCACCAGUGUCCA	376
2811	UACCUAAAGCAGUCUAUU	157	2811	UACCUAAAGCAGUCUAUU	157	2829	AAAUAGACUGCUUUAAGUA	377
2829	UAUAUUUCUACAUUAU	158	2829	UAUAUUUCUACAUUAU	158	2847	AUUAGAUUGAAGAAUUA	378
2847	UUUUAAGAGCCUGGCUACA	159	2847	UUUUAAGAGCCUGGCUACA	159	2865	UGUAGCCAGGCUUUAUUA	379
2865	AAUACUGCACAACUUGGU	160	2865	AAUACUGCACAACUUGGU	160	2883	ACCAAGUUUUGUGCAGUAUU	380
2883	UUAGUCAAUUUUUGAUCC	161	2883	UUAGUCAAUUUUUGAUCC	161	2901	GGAUCAAAAAUUUGAACUAA	381
2901	CCUUUCUACUUAUUUAAC	162	2901	CCUUUCUACUUAUUUAAC	162	2919	GUAAAAUUAAGUAGAAAGGG	382
2919	CAUUAAGCUCUUAUUUAAG	163	2919	CAUUAAGCUCUUAUUUAAG	163	2937	CUAAAAAGAGCAUUAUUG	383
2937	GUAGUUCUUAUUAUGCUGG	164	2937	GUAGUUCUUAUUAUGCUGG	164	2955	CCAGCAUUAAGAACAUAC	384

2955	GAUCACAGACAGCUCUAUU	165	2955	GAUCACAGACAGCUCUAUU	165	2973	AAUAGAGCUCUCUGUGAUC	385
2973	UUCUCAGUUUUUUGGUUU	166	2973	UUCUCAGUUUUUUGGUUU	166	2991	AUACCAAAACACUGAGAA	386
2991	UUAACCAUUGCAUUGCAG	167	2991	UUAACCAUUGCAUUGCAG	167	3009	CUGCAUUGCAUUGGUUAA	387
3009	GUAGCAUUAUUUUAAAAA	168	3009	GUAGCAUUAUUUUAAAAA	168	3027	UUUUUUAAAAUAGUGUAC	388
3027	AUGCACUUUUUUUUUUUU	169	3027	AUGCACUUUUUUUUUUUU	169	3045	AAUAAUUAAAAAGGUGCAU	389
3045	UUUUUUUUUGGCUAGGGAGU	170	3045	UUUUUUUUUGGCUAGGGAGU	170	3063	ACUCCUAGCCCAAAAUAA	390
3063	UUUAUCCUUUUUUCGAAUU	171	3063	UUUAUCCUUUUUUCGAAUU	171	3081	AAUUCGAAAAAGGGAAUAA	391
3081	UAUUUUUAAGAAUGCCA	172	3081	UAUUUUUAAGAAUGCCA	172	3099	UGGCAUUCUUUUAAAAUUA	392
3099	AAUAUAUUUUUGUAAGAA	173	3099	AAUAUAUUUUUGUAAGAA	173	3117	UUUUUACAAAAUUUAUUU	393
3117	AGGCAGUAACCUUUAUCA	174	3117	AGGCAGUAACCUUUAUCA	174	3135	UGAUGAAAGGUUACUGCCU	394
3135	AUGAUCUAGGCAGUUUGAA	175	3135	AUGAUCUAGGCAGUUUGAA	175	3153	UUAACUGCCUUAUGAUCAU	395
3153	AAAAUUUUUACACCUUUUU	176	3153	AAAAUUUUUACACCUUUUU	176	3171	AAAAGGUGUAAAAAUUUU	396
3171	UUUUCACAUUUUAUUAUAA	177	3171	UUUUCACAUUUUAUUAUAA	177	3189	UUUAUGUAAAAUGUGAAAA	397
3189	AUAUAUUGCUUUGCCAGC	178	3189	AUAUAUUGCUUUGCCAGC	178	3207	GCUGGCAAGCAUUUAUUU	398
3207	CAGUACGUGGUAAGCCACAA	179	3207	CAGUACGUGGUAAGCCACAA	179	3225	UUUGGCUACCCAGUACUG	399
3225	AUUGCACAUUAUUUUUCU	180	3225	AUUGCACAUUAUUUUUCU	180	3243	AGAAAAUUAUUUGUGCAU	400
3243	UUAAAAUACCCAGCAGUU	181	3243	UUAAAAUACCCAGCAGUU	181	3261	AACUGCUGGUUUUUUUUAA	401
3261	UACUAGGAAUAUAUUUCU	182	3261	UACUAGGAAUAUAUUUCU	182	3279	AGAAUAUAUCCUAGUGUA	402
3279	UGCGUUUAUAAAAUAGUU	183	3279	UGCGUUUAUAAAAUAGUU	183	3297	AACUAGUUUUUAUAAACGCA	403
3297	UUUUAAGAAAGAAUUUUUU	184	3297	UUUUAAGAAAGAAUUUUUU	184	3315	AAAAUUUUUCUUCUUAUAA	404
3315	UUUGGCCUUAUAAUUUGUU	185	3315	UUUGGCCUUAUAAUUUGUU	185	3333	AACAAUUUUAUAGGCCAAA	405
3333	UAAACCUUGAACAUAGACAU	186	3333	UAAACCUUGAACAUAGACAU	186	3351	AUGUCAUGUCCAGGUUUA	406
3351	UUGUUAUUAUAUAUAUAU	187	3351	UUGUUAUUAUAUAUAUAU	187	3369	AUUUUUAUUAUUAUAAUAA	407
3369	UGAUUCUUAUUAUUGCUGAU	188	3369	UGAUUCUUAUUAUUGCUGAU	188	3387	AUACAGCAUUUAAGAAUUA	408
3387	UGGUUUUAUUAUUUAAUUG	189	3387	UGGUUUUAUUAUUUAAUUG	189	3405	CCAUUUUAUUAUAAUAAUAA	409
3405	GGUAAAGCCAUUUUAUUA	190	3405	GGUAAAGCCAUUUUAUUA	190	3423	UUUAUGUAAUUGGCUUUAAC	410
3423	AUAUAGAAAGAUUAUGCAU	191	3423	AUAUAGAAAGAUUAUGCAU	191	3441	UAUGCAUAUCUUAUUAUUA	411
3441	AUAUCUAGAGGUUAUGUGG	192	3441	AUAUCUAGAGGUUAUGUGG	192	3459	CCACAUACCUUUAUUAUUA	412
3459	GCAUUUAUUUGGAUAAAAU	193	3459	GCAUUUAUUUGGAUAAAAU	193	3477	AUUUUUCCAAUUAUAAUUGC	413
3477	UUCUCAUUUCAGAGAAUUC	194	3477	UUCUCAUUUCAGAGAAUUC	194	3495	GAUUUCUCUGAAUUAUUAUUA	414
3495	CAUCUGAUUUUUAUUAUUA	195	3495	CAUCUGAUUUUUAUUAUUA	195	3513	ACUAUAGAAACAUCAUUAUG	415
3513	UCACUUUUGCCAGCUCUAAA	196	3513	UCACUUUUGCCAGCUCUAAA	196	3531	UUUUGAGCUGGCCAAAGUGA	416
3531	AGAAAAUUAUACCCUUAUGU	197	3531	AGAAAAUUAUACCCUUAUGU	197	3549	ACAUAGGGUUAUUGUUUUCU	417
3549	UAGUUUGGGAAGUUUAUUGC	198	3549	UAGUUUGGGAAGUUUAUUGC	198	3567	GCAUAAACUCCACAAUUA	418

3567	CUAUAUUGUGUAACUGAU	199	3567	CUAUAUUGUGUAACUGAU	199	3585	AUCAGUUACACAAUUAUAG	419
3585	UAUUAACCUAAUUGUUCU	200	3585	UAUUAACCUAAUUGUUCU	200	3603	AGAACAUIUAGGUUUAUA	420
3603	UGCCUACCCUGUUGGUUA	201	3603	UGCCUACCCUGUUGGUUA	201	3621	UAUACCAACAGGGUAGGCA	421
3621	AAAGAUUUUUGAGCAGAC	202	3621	AAAGAUUUUUGAGCAGAC	202	3639	GUCUGCUCAAAAUUCUUIU	422
3639	CUGUAAACAAGAAAAAAA	203	3639	CUGUAAACAAGAAAAAAA	203	3657	UUUUUUUUCUUGUUUACAG	423
3657	AAAUCAUGCAUUCUUAGCA	204	3657	AAAUCAUGCAUUCUUAGCA	204	3675	UGCUAAGAAUGCAUGAUUIU	424
3675	AAAUUGCCUAGUAUGUUA	205	3675	AAAUUGCCUAGUAUGUUA	205	3693	UAACAUACUAGGCAAUUUU	425
3693	AAUUGCUCAAAAUACAAU	206	3693	AAUUGCUCAAAAUACAAU	206	3711	AUUGUAUUUUUGAGCAAUUU	426
3711	UGUUUGAUUUUUAUGCAGUU	207	3711	UGUUUGAUUUUUAUGCAGUU	207	3729	AAGUGCAUAAAAUCAAACA	427
3729	UUGUCGCUAUUAACAUCUU	208	3729	UUGUCGCUAUUAACAUCUU	208	3747	AGGAUGUUUAUAGCGACAA	428
3747	UUUUUUUUCAUUGAGAUUUC	209	3747	UUUUUUUUCAUUGAGAUUUC	209	3765	GAAAUCAUAGUAGAAAAA	429
3765	CAUAUAUUGAGUAUUUUA	210	3765	CAUAUAUUGAGUAUUUUA	210	3783	UAAAUAUACUCAAUUAUUG	430
3783	AGAAAGCAUUAUUUAGGAA	211	3783	AGAAAGCAUUAUUUAGGAA	211	3801	UUCUAAAAUAAUGCUUCU	431
3801	AUAUAUAGUUUGACACAGUA	212	3801	AUAUAUAGUUUGACACAGUA	212	3819	UACUGUGACAACUAUAUAU	432
3819	AAUAUCUUGUUUUUUCUA	213	3819	AAUAUCUUGUUUUUUCUA	213	3837	UAGAAAAACAAGAUUAUUU	433
3837	AUGUACAUUGUACAAAUUU	214	3837	AUGUACAUUGUACAAAUUU	214	3855	AAAUUUGUACAAUUGUACAU	434
3855	UUUCAUUCUUUUUGCUCUU	215	3855	UUUCAUUCUUUUUGCUCUU	215	3873	AAGAGCAAAAGGAUUGAAA	435
3873	UUGUGGUUGGAUCUACAC	216	3873	UUGUGGUUGGAUCUACAC	216	3891	GUGUUAUAGUCCAACCCAA	436
3891	CUAACUGUAUUUUUGUUU	217	3891	CUAACUGUAUUUUUGUUU	217	3909	AACAAAACAUAACAGUUUAG	437
3909	UACAUCAAAUAACAUCUU	218	3909	UACAUCAAAUAACAUCUU	218	3927	AAGAUGUUUUUUUGAUGUA	438
3927	UCUGUGGACCAGGAAAAAA	219	3927	UCUGUGGACCAGGAAAAAA	219	3945	UUUUUCCUGGUCCACAGA	439
3932	GGACCAGGAAAAAA	220	3932	GGACCAGGAAAAAA	220	3950	UUUUUUUUUUUCCUGGUCC	440

The 3'-ends of the Upper sequence and the Lower sequence of the siNA construct can include an overhang sequence, for example about 1, 2, 3, or 4 nucleotides in length, preferably 2 nucleotides in length, wherein the overhanging sequence of the lower sequence is optionally complementary to a portion of the target sequence. The upper sequence is also referred to as the sense strand, whereas the lower sequence is also referred to as the antisense strand. The upper and lower sequences in the Table can further comprise a chemical modification having Formulae I-VII, such as exemplary siNA constructs shown in Figures 4 and 5, or having modifications described in Table IV or any combination thereof.

03-806-A; 400/201

TABLE III: HIF1 Synthetic Modified siNA Constructs

Target Pos	Target	Seq ID	Cmpd#	Aliases	Sequence	Seq ID
175	CUCUGGACUUGCCUUAUCCUUCUC	441		HIF1:177U21 sense siNA	CUGGACUUGCCUUAUCCUUCCTT	449
440	GCAUCUUGAUAAAGGCCUCUGUGA	442		HIF1:442U21 sense siNA	AUCUUGAUAAAGGCCUCUGUUTT	450
495	AAACUUCUGGAUGCUGGUGAUUU	443		HIF1:497U21 sense siNA	ACUUCUGGAUGCUGGUGAUUTT	451
1068	UUGAUGGGAUUAUGAGCCAGAAGA	444		HIF1:1070U21 sense siNA	GAUGGGAUUAUGAGCCAGAATT	452
1097	AGGCCGCUCAAUUAUGAAUAUU	445		HIF1:1099U21 sense siNA	GCCGCUCAAUUAUGAAUAATT	453
1771	AUCAGACACCUAGUCCUCCGAU	446		HIF1:1773U21 sense siNA	CAGACACCUAGUCCUCCGTT	454
2009	UUCUUCGAUCAGUUGACACCAU	447		HIF1:2011U21 sense siNA	CCUUCGAUCAGUUGACACCTT	455
2135	CACUGCCACCAUCUGAUAUAUA	448		HIF1:2137U21 sense siNA	CUGCCACCAUCUGAUAUAUTT	456
175	CUCUGGACUUGCCUUAUCCUUCUC	441		HIF1:195L21 antisense siNA (177C)	GAAGGAAAGGCAAGUCCAGTT	457
440	GCAUCUUGAUAAAGGCCUCUGUGA	442		HIF1:460L21 antisense siNA (442C)	ACAGAGGCCUUAUCAAGAATT	458
495	AAACUUCUGGAUGCUGGUGAUUU	443		HIF1:515L21 antisense siNA (497C)	AUCACAGCAUCCAGCAAGUUTT	459
1068	UUGAUGGGAUUAUGAGCCAGAAGA	444		HIF1:1088L21 antisense siNA (1070C)	UUCUGGCUCAUAUCCCAUUCTT	460
1097	AGGCCGCUCAAUUAUGAAUAUU	445		HIF1:1117L21 antisense siNA (1099C)	UAUUCAUAAUAUAGCGCGCTT	461
1771	AUCAGACACCUAGUCCUCCGAU	446		HIF1:1791L21 antisense siNA (1773C)	CGGAAGGACUAGGUGUCUGTT	462
2009	UUCUUCGAUCAGUUGACACCAU	447		HIF1:2029L21 antisense siNA (2011C)	GGUGACAACUGAUCGAAAGTT	463
2135	CACUGCCACCAUCUGAUAUAUA	448		HIF1:2155L21 antisense siNA (2137C)	AAUUCACAGUGGUGGCGAGTT	464
175	CUCUGGACUUGCCUUAUCCUUCUC	441		HIF1:177U21 sense siNA stab04	B CUGGACUUGCCUUAUCCUUCCTT B	465
440	GCAUCUUGAUAAAGGCCUCUGUGA	442		HIF1:442U21 sense siNA stab04	B AUCUUGAUAAAGGCCUUGUUTT B	466
495	AAACUUCUGGAUGCUGGUGAUUU	443		HIF1:497U21 sense siNA stab04	B ACUUCUGGAUGCUGGUGAUUTT B	467
1068	UUGAUGGGAUUAUGAGCCAGAAGA	444		HIF1:1070U21 sense siNA stab04	B GAUGGGAUUAUGAGCCAGAATT B	468
1097	AGGCCGCUCAAUUAUGAAUAUU	445		HIF1:1099U21 sense siNA stab04	B GCGCUCUAAUUAUGAAUAATT B	469
1771	AUCAGACACCUAGUCCUCCGAU	446		HIF1:1773U21 sense siNA stab04	B CAGACACCUAGUCCUCCGTT B	470
2009	UUCUUCGAUCAGUUGACACCAU	447		HIF1:2011U21 sense siNA stab04	B CCUUCGAUCAGUUGACACCTT B	471
2135	CACUGCCACCAUCUGAUAUAUA	448		HIF1:2137U21 sense siNA stab04	B CUGCCACCAUCUGAUAUAUTT B	472
175	CUCUGGACUUGCCUUAUCCUUCUC	441		HIF1:195L21 antisense siNA (177C) stab05	GAAGGAAAGGCAAGUCCAGTsT	473
440	GCAUCUUGAUAAAGGCCUCUGUGA	442		HIF1:460L21 antisense siNA (442C) stab05	AcAGAGGCCUUAUCAAGAUtsT	474
495	AAACUUCUGGAUGCUGGUGAUUU	443		HIF1:515L21 antisense siNA (497C) stab05	AucAccAGCAUCCAGAAGuTsT	475
1068	UUGAUGGGAUUAUGAGCCAGAAGA	444		HIF1:1088L21 antisense siNA (1070C) stab05	uuuGCGUCUUAUCCCAUcTsT	476
1097	AGGCCGCUCAAUUAUGAAUAUU	445		HIF1:1117L21 antisense siNA (1099C) stab05	uAUUCAUAAAUUAGCGCGTsT	477

03-806-A; 400/201

1771	AUCAGACACCUAGUCCUUCGGAU	446		HIF1:1791L21 antisense siNA (1773C) stab05	cGGAAGGAcuAGGuGucUGTsT	478
2009	UUCUUCGACUAGUUGUCACCAU	447		HIF1:2029L21 antisense siNA (2011C) stab05	GGUGAcAAcuGAcuGAAGGTsT	479
2135	CACUGCCACCAUCUGAAUUA	448		HIF1:2155L21 antisense siNA (2137C) stab05	AAuucAucAGuGGuGGcAGTsT	480
175	CUCUGGACUUGCCUUCUUCUC	441		HIF1:177U21 sense siNA stab07	B cuGGAcuuGccuuuccuucTT B	481
440	GCAUCUUGAUAGGCCUCUGUGA	442		HIF1:442U21 sense siNA stab07	B AuuuuGAAuAGGccuucGUtt B	482
485	AAACUUCUGGAGUGGUGAUUU	443		HIF1:497U21 sense siNA stab07	B AuuuuGGAuGGuGGuGAUtt B	483
1088	UUGAUGGGAUUGAGCCAGAAGA	444		HIF1:1070U21 sense siNA stab07	B GAUGGGGAuAGGccAGAAtt B	484
1097	AGGCGCUCUAAUUAUGAAUUAU	445		HIF1:1098U21 sense siNA stab07	B GccGcuAAuuuAuGAuuATT B	485
1771	AUCAGACACCUAGUCCUUCGGAU	446		HIF1:1773U21 sense siNA stab07	B cAGAcAcuuAGuccuuccGTT B	486
2009	UUCUUCGACUAGUUGUCACCAU	447		HIF1:2011U21 sense siNA stab07	B ccuuuGAcuAGuuGucAccTT B	487
2135	CACUGCCACCAUCUGAAUUA	448		HIF1:2137U21 sense siNA stab07	B cuGccAcAcuGAcuGAuuTT B	488
175	CUCUGGACUUGCCUUCUUCUC	441		HIF1:195L21 antisense siNA (177C) stab11	GAAGGAAAGGcAAGuccAGTsT	489
440	GCAUCUUGAUAGGCCUCUGUGA	442		HIF1:460L21 antisense siNA (442C) stab11	AcAGAGGccuuuAucAAGAUtsT	490
485	AAACUUCUGGAGUGGUGAUUU	443		HIF1:515L21 antisense siNA (497C) stab11	AucAccAGcAuccAAGAAGuTsT	491
1088	UUGAUGGGAUUGAGCCAGAAGA	444		HIF1:1088L21 antisense siNA (1070C) stab11	uuuuGGcuuAuuuuuAucTsT	492
1097	AGGCGCUCUAAUUAUGAAUUAU	445		HIF1:1117L21 antisense siNA (1089C) stab11	uAuuuAuuAAuuuGAGcGGcTsT	493
1771	AUCAGACACCUAGUCCUUCGGAU	446		HIF1:1791L21 antisense siNA (1773C) stab11	cGGAAGGAcuAGGuGucUGTsT	494
2009	UUCUUCGACUAGUUGUCACCAU	447		HIF1:2029L21 antisense siNA (2011C) stab11	GGUGAcAAcuGAcuGAAGGTsT	495
2135	CACUGCCACCAUCUGAAUUA	448		HIF1:2155L21 antisense siNA (2137C) stab11	AAuucAucAGuGGuGGcAGTsT	496
175	CUCUGGACUUGCCUUCUUCUC	441		HIF1:177U21 sense siNA stab18	B cuGGAcuuGccuuuccuucTT B	497
440	GCAUCUUGAUAGGCCUCUGUGA	442		HIF1:442U21 sense siNA stab18	B AuuuuGAAuAGGccuucGUtt B	498
485	AAACUUCUGGAGUGGUGAUUU	443		HIF1:497U21 sense siNA stab18	B AuuuuGGAuGGuGGuGAUtt B	499
1088	UUGAUGGGAUUGAGCCAGAAGA	444		HIF1:1070U21 sense siNA stab18	B GAUGGGGAuAGGccAGAAtt B	500
1097	AGGCGCUCUAAUUAUGAAUUAU	445		HIF1:1098U21 sense siNA stab18	B GccGcuAAuuuAuGAuuATT B	501
1771	AUCAGACACCUAGUCCUUCGGAU	446		HIF1:1773U21 sense siNA stab18	B cAGAcAcuuAGuccuuccGTT B	502
2009	UUCUUCGACUAGUUGUCACCAU	447		HIF1:2011U21 sense siNA stab18	B ccuuuGAcuAGuuGucAccTT B	503
2135	CACUGCCACCAUCUGAAUUA	448		HIF1:2137U21 sense siNA stab18	B cuGccAcAcuGAcuGAuuTT B	504
175	CUCUGGACUUGCCUUCUUCUC	441	33621	HIF1:195L21 antisense siNA (177C) stab08	GAAGGAAAGGcAAGuccAGTsT	505

03-806-A; 400/201

440	GCAUCUUGAUAAAGCCUCUCUGA	442	33622	HIF1:460L21 antisense s1NA (442C) stab08	AcAGAGGGccuuAucAAAGAUtTsT	508
495	AAACUUCUGGAUGCUGGUGAUUU	443	33623	HIF1:515L21 antisense s1NA (497C) stab08	AucAccAGcAuccAGAAAGUtsT	507
1088	UUGAUGGGAUUAUGAGCCAGAAGA	444	33624	HIF1:1088L21 antisense s1NA (1070C) stab08	uuuuGGcucAuAucccAucTsT	508
1087	AGCCGCGUCAUUUAUGAAUAUU	445	33625	HIF1:1117L21 antisense s1NA (1089C) stab08	uAuuCAuAAAuuGAGcGcTsT	509
1771	AUCAGACACCUAGUCCUUCCGAU	446	33626	HIF1:1791L21 antisense s1NA (1773C) stab08	cGGAAGGgAcuAGGUgucUgTsT	510
2009	UUCUUCGAUCAGUUGUCACCAU	447	33627	HIF1:2029L21 antisense s1NA (2011C) stab08	GGuGAcAAcuGAucGAAGGTsT	511
2135	CACUGCCACCACUGAUGAAUUA	448	33628	HIF1:2155L21 antisense s1NA (2137C) stab08	AAuucAucAGuGGuGGcAGTsT	512
175	CUCUGGACUUGCCUUCUUCUC	441	33605	HIF1:177U21 sense s1NA stab09	B CUGGACUUGCCUUCUUCUUTT B	513
440	GCAUCUUGAUAAAGCCUCUGA	442	33608	HIF1:442U21 sense s1NA stab09	B AUCUUGAUAAAGCCUCUGUTT B	514
495	AAACUUCUGGAUGCUGGUGAUUU	443	33607	HIF1:497U21 sense s1NA stab09	B ACUUCUGGAUGCUGGUGAUTT B	515
1088	UUGAUGGGAUUAUGAGCCAGAAGA	444	33608	HIF1:1070U21 sense s1NA stab09	B GAUGGGAUAUGAGCCAGAAATT B	516
1087	AGCCGCGUCAUUUAUGAAUAUU	445	33609	HIF1:1099U21 sense s1NA stab09	B GCCGCUCAAUUUAUGAAUATT B	517
1771	AUCAGACACCUAGUCCUUCCGAU	446	33610	HIF1:1773U21 sense s1NA stab09	B CAGACACCUAGUCCUUCCGTT B	518
2009	UUCUUCGAUCAGUUGUCACCAU	447	33611	HIF1:2011U21 sense s1NA stab09	B CCUUCGAUCAGUUGUCACCTT B	519
2135	CACUGCCACCACUGAUGAAUUA	448	33612	HIF1:2137U21 sense s1NA stab09	B CUGCCACCACUGAUGAAUUTT B	520
175	CUCUGGACUUGCCUUCUUCUC	441	33613	HIF1:195L21 antisense s1NA (177C) stab10	GAAGGAAAAGcAAAGUCCAGTsT	521
440	GCAUCUUGAUAAAGCCUCUGA	442	33614	HIF1:460L21 antisense s1NA (442C) stab10	ACAGAGGGCCUUAUCAAGAUtsT	522
495	AAACUUCUGGAUGCUGGUGAUUU	443	33615	HIF1:515L21 antisense s1NA (497C) stab10	AUCACCAGCAUCCAGAAAGUtsT	523
1088	UUGAUGGGAUUAUGAGCCAGAAGA	444	33616	HIF1:1088L21 antisense s1NA (1070C) stab10	UUCUGGCUCAUUAUCCCAUctTsT	524
1087	AGCCGCGUCAUUUAUGAAUAUU	445	33617	HIF1:1117L21 antisense s1NA (1089C) stab10	UAUUCAUAAAUUAGCGGCTsT	525
1771	AUCAGACACCUAGUCCUUCCGAU	446	33618	HIF1:1791L21 antisense s1NA (1773C) stab10	CGGAAGGACUAGGUGUCUGTsT	526
2009	UUCUUCGAUCAGUUGUCACCAU	447	33619	HIF1:2029L21 antisense s1NA (2011C) stab10	GGUGACAACUGAUCGAAAGGTsT	527
2135	CACUGCCACCACUGAUGAAUUA	448	33620	HIF1:2155L21 antisense s1NA (2137C) stab10	AAUUCAUACAGUGGUGGCAGTsT	528
175	CUCUGGACUUGCCUUCUUCUC	441		HIF1:195L21 antisense s1NA (177C) stab19	GAAAGAAAGGcAAAGuccAGTT B	529
440	GCAUCUUGAUAAAGCCUCUGA	442		HIF1:460L21 antisense s1NA (442C)	AcAGAGGGccuuAucAAAGAUtT B	530

03-806-A; 400/201

495	AAACUUCUGGAUGCUGGUGAUUU	443	stab18		
1068	UUGAUGGGGAUAGAGCCAGAAGA	444	HIF1:515L21 antisense siNA (497C) stab18	AUCACcAGcAUccAGAAgUtt B	531
1097	AGCCGCUCAAUUUUAUGAAUAAU	445	HIF1:1088L21 antisense siNA (1070C) stab18	UUCUGGcUcAUuAUccAUcTt B	532
1771	AUCAGACACCUAGUCCUUCCGAU	446	HIF1:117L21 antisense siNA (1089C) stab19	UAUUCAUAAAUuGAGcGGcTt B	533
2009	UUCUUCGAUCAGUUGUCACCAU	447	HIF1:1781L21 antisense siNA (1773C) stab19	cGGAAGGAGcUAGGUgUcUgTt B	534
2135	CACUGCCACCACUGAUGAAUAA	448	HIF1:2028L21 antisense siNA (2011C) stab19	GGUGAcAAcUgAUcGAAgGtT B	535
175	CUCUGGACUUGCCUUCUUCUC	441	HIF1:2155L21 antisense siNA (2137C) stab19	AAUUCAUcAGUGGUgGcAGTt B	536
440	GCAUCUUGAUAAGGCCUUCUGUGA	442	HIF1:195L21 antisense siNA (177C) stab22	GAAGGAAGGCAAGUCCAGTt B	537
495	AAACUUCUGGAUGCUGGUGAUUU	443	HIF1:460L21 antisense siNA (442C) stab22	ACAGAGGCCUUUAUCAAGAUtT B	538
1068	UUGAUGGGGAUAGAGCCAGAAGA	444	HIF1:515L21 antisense siNA (497C) stab22	AUCACCAGCAUCCAGAAgUtt B	539
1097	AGCCGCUCAAUUUUAUGAAUAAU	445	HIF1:1088L21 antisense siNA (1070C) stab22	UUCUGGCUCAUuAUCCAUcTt B	540
1771	AUCAGACACCUAGUCCUUCCGAU	446	HIF1:117L21 antisense siNA (1089C) stab22	UAUUCAUAAAUUGAGCGGcTt B	541
2009	UUCUUCGAUCAGUUGUCACCAU	447	HIF1:1781L21 antisense siNA (1773C) stab22	CGGAAGGACUAGGUGUCUGTt B	542
2135	CACUGCCACCACUGAUGAAUAA	448	HIF1:2028L21 antisense siNA (2011C) stab22	GGUGACAACUGAUcGAAgGtT B	543
			HIF1:2155L21 antisense siNA (2137C) stab22	AAUUCAUcAGUGGUgGcAGTt B	544

Uppercase = ribonucleotide
u,c = 2'-deoxy-2'-fluoro U,C
T = thymidine
B = inverted deoxy abasic
s = phosphorothioate linkage
A = deoxy Adenosine
G = deoxy Guanosine
G = 2'-O-methyl Guanosine
A = 2'-O-methyl Adenosine

Table IV

Non-limiting examples of Stabilization Chemistries for chemically modified siRNA constructs

Chemistry	pyrimidine	Purine	cap	p=S	Strand
"Stab 00"	Ribo	Ribo	TT at 3'-ends		S/AS
"Stab 1"	Ribo	Ribo	-	5 at 5'-end 1 at 3'-end	S/AS
"Stab 2"	Ribo	Ribo	-	All linkages	Usually AS
"Stab 3"	2'-fluoro	Ribo	-	4 at 5'-end 4 at 3'-end	Usually S
"Stab 4"	2'-fluoro	Ribo	5' and 3'-ends	-	Usually S
"Stab 5"	2'-fluoro	Ribo	-	1 at 3'-end	Usually AS
"Stab 6"	2'-O-Methyl	Ribo	5' and 3'-ends	-	Usually S
"Stab 7"	2'-fluoro	2'-deoxy	5' and 3'-ends	-	Usually S
"Stab 8"	2'-fluoro	2'-O-Methyl	-	1 at 3'-end	S/AS
"Stab 9"	Ribo	Ribo	5' and 3'-ends	-	Usually S
"Stab 10"	Ribo	Ribo	-	1 at 3'-end	Usually AS
"Stab 11"	2'-fluoro	2'-deoxy	-	1 at 3'-end	Usually AS
"Stab 12"	2'-fluoro	LNA	5' and 3'-ends		Usually S
"Stab 13"	2'-fluoro	LNA		1 at 3'-end	Usually AS
"Stab 14"	2'-fluoro	2'-deoxy		2 at 5'-end 1 at 3'-end	Usually AS
"Stab 15"	2'-deoxy	2'-deoxy		2 at 5'-end 1 at 3'-end	Usually AS
"Stab 16"	Ribo	2'-O-Methyl	5' and 3'-ends		Usually S
"Stab 17"	2'-O-Methyl	2'-O-Methyl	5' and 3'-ends		Usually S
"Stab 18"	2'-fluoro	2'-O-Methyl	5' and 3'-ends		Usually S
"Stab 19"	2'-fluoro	2'-O-Methyl	3'-end		S/AS
"Stab 20"	2'-fluoro	2'-deoxy	3'-end		Usually AS
"Stab 21"	2'-fluoro	Ribo	3'-end		Usually AS
"Stab 22"	Ribo	Ribo	3'-end		Usually AS
"Stab 23"	2'-fluoro*	2'-deoxy*	5' and 3'-ends		Usually S
"Stab 24"	2'-fluoro*	2'-O-Methyl*	-	1 at 3'-end	S/AS
"Stab 25"	2'-fluoro*	2'-O-Methyl*	-	1 at 3'-end	S/AS

"Stab 26"	2'-fluoro*	2'-O-Methyl*	-		S/AS
"Stab 27"	2'-fluoro*	2'-O-Methyl*	3'-end		S/AS
"Stab 28"	2'-fluoro*	2'-O-Methyl*	3'-end		S/AS
"Stab 29"	2'-fluoro*	2'-O-Methyl*		1 at 3'-end	S/AS
"Stab 30"	2'-fluoro*	2'-O-Methyl*			S/AS
"Stab 31"	2'-fluoro*	2'-O-Methyl*	3'-end		S/AS
"Stab 32"	2'-fluoro	2'-O-Methyl			S/AS

CAP = any terminal cap, see for example Figure 10.

All Stab 00-32 chemistries can comprise 3'-terminal thymidine (TT) residues

All Stab 00-32 chemistries typically comprise about 21 nucleotides, but can vary as described herein.

S = sense strand

AS = antisense strand

*Stab 23 has a single ribonucleotide adjacent to 3'-CAP

*Stab 24 and Stab 28 have a single ribonucleotide at 5'-terminus

*Stab 25, Stab 26, and Stab 27 have three ribonucleotides at 5'-terminus

*Stab 29, Stab 30, and Stab 31, any purine at first three nucleotide positions from 5'-terminus are ribonucleotides

p = phosphorothioate linkage

Table V

A. 2.5 μ mol Synthesis Cycle ABI 394 Instrument

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time*RNA
Phosphoramidites	6.5	163 μ L	45 sec	2.5 min	7.5 min
S-Ethyl Tetrazole	23.8	238 μ L	45 sec	2.5 min	7.5 min
Acetic Anhydride	100	233 μ L	5 sec	5 sec	5 sec
N-Methyl Imidazole	188	233 μ L	5 sec	5 sec	5 sec
TCA	176	2.3 mL	21 sec	21 sec	21 sec
Iodine	11.2	1.7 mL	45 sec	45 sec	45 sec
Beaucage	12.9	645 μ L	100 sec	300 sec	300 sec
Acetonitrile	NA	6.67 mL	NA	NA	NA

B. 0.2 μ mol Synthesis Cycle ABI 394 Instrument

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time*RNA
Phosphoramidites	15	31 μ L	45 sec	233 sec	465 sec
S-Ethyl Tetrazole	38.7	31 μ L	45 sec	233 min	465 sec
Acetic Anhydride	655	124 μ L	5 sec	5 sec	5 sec
N-Methyl Imidazole	1245	124 μ L	5 sec	5 sec	5 sec
TCA	700	732 μ L	10 sec	10 sec	10 sec
Iodine	20.6	244 μ L	15 sec	15 sec	15 sec
Beaucage	7.7	232 μ L	100 sec	300 sec	300 sec
Acetonitrile	NA	2.64 mL	NA	NA	NA

C. 0.2 μ mol Synthesis Cycle 96 well Instrument

Reagent	Equivalents:DNA/ 2'-O-methyl/Ribo	Amount: DNA/2'-O- methyl/Ribo	Wait Time* DNA	Wait Time* 2'-O- methyl	Wait Time* Ribo
Phosphoramidites	22/33/66	40/60/120 μ L	60 sec	180 sec	360sec
S-Ethyl Tetrazole	70/105/210	40/60/120 μ L	60 sec	180 min	360 sec
Acetic Anhydride	265/265/265	50/50/50 μ L	10 sec	10 sec	10 sec
N-Methyl Imidazole	502/502/502	50/50/50 μ L	10 sec	10 sec	10 sec
TCA	238/475/475	250/500/500 μ L	15 sec	15 sec	15 sec
Iodine	6.8/6.8/6.8	80/80/80 μ L	30 sec	30 sec	30 sec
Beaucage	34/51/51	80/120/120	100 sec	200 sec	200 sec
Acetonitrile	NA	1150/1150/1150 μ L	NA	NA	NA

- 5
- Wait time does not include contact time during delivery.
 - Tandem synthesis utilizes double coupling of linker molecule

CLAIMS

What we claim is:

1. A chemically synthesized double stranded short interfering nucleic acid (siNA) molecule that directs cleavage of a HIF1 RNA via RNA interference (RNAi), wherein:
 - a) each strand of said siNA molecule is about 18 to about 23 nucleotides in length; and
 - b) one strand of said siNA molecule comprises nucleotide sequence having sufficient complementarity to said HIF1 RNA for the siNA molecule to direct cleavage of the HIF1 RNA via RNA interference.
2. The siNA molecule of claim 1, wherein said siNA molecule comprises no ribonucleotides.
3. The siNA molecule of claim 1, wherein said siNA molecule comprises one or more ribonucleotides.
4. The siNA molecule of claim 1, wherein one strand of said double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence of a HIF1 gene or a portion thereof, and wherein a second strand of said double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence or a portion thereof of said HIF1 RNA.
5. The siNA molecule of claim 4, wherein each strand of the siNA molecule comprises about 18 to about 23 nucleotides, and wherein each strand comprises at least about 19 nucleotides that are complementary to the nucleotides of the other strand.
6. The siNA molecule of claim 1, wherein said siNA molecule comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence of a HIF1 gene or a portion thereof, and wherein said siNA further comprises a sense region, wherein said sense region comprises a nucleotide sequence substantially similar to the nucleotide sequence of said HIF1 gene or a portion thereof.
7. The siNA molecule of claim 6, wherein said antisense region and said sense region comprise about 18 to about 23 nucleotides, and wherein said antisense region

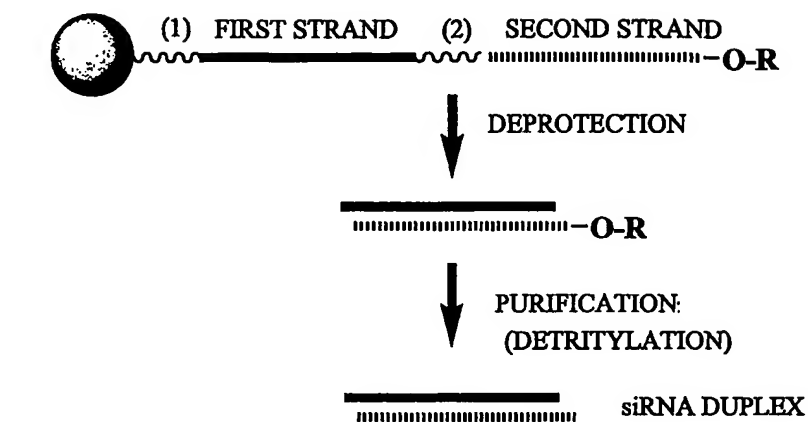
comprises at least about 18 nucleotides that are complementary to nucleotides of the sense region.

8. The siNA molecule of claim 1, wherein said siNA molecule comprises a sense region and an antisense region, and wherein said antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by a HIF1 gene, or a portion thereof, and said sense region comprises a nucleotide sequence that is complementary to said antisense region.
9. The siNA molecule of claim 6, wherein said siNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and a second fragment comprises the antisense region of said siNA molecule.
10. The siNA molecule of claim 6, wherein said sense region is connected to the antisense region via a linker molecule.
11. The siNA molecule of claim 10, wherein said linker molecule is a polynucleotide linker.
12. The siNA molecule of claim 10, wherein said linker molecule is a non-nucleotide linker.
13. The siNA molecule of claim 6, wherein pyrimidine nucleotides in the sense region are 2'-O-methyl pyrimidine nucleotides.
14. The siNA molecule of claim 6, wherein purine nucleotides in the sense region are 2'-deoxy purine nucleotides.
15. The siNA molecule of claim 6, wherein pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides.
16. The siNA molecule of claim 9, wherein the fragment comprising said sense region includes a terminal cap moiety at a 5'-end, a 3'-end, or both of the 5' and 3' ends of the fragment comprising said sense region.
17. The siNA molecule of claim 16, wherein said terminal cap moiety is an inverted deoxy abasic moiety.
18. The siNA molecule of claim 6, wherein pyrimidine nucleotides of said antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides.

19. The siNA molecule of claim 6, wherein purine nucleotides of said antisense region are 2'-O-methyl purine nucleotides.
20. The siNA molecule of claim 6, wherein purine nucleotides present in said antisense region comprise 2'-deoxy- purine nucleotides.
21. The siNA molecule of claim 18, wherein said antisense region comprises a phosphorothioate internucleotide linkage at the 3' end of said antisense region.
22. The siNA molecule of claim 6, wherein said antisense region comprises a glyceryl modification at a 3' end of said antisense region.
23. The siNA molecule of claim 9, wherein each of the two fragments of said siNA molecule comprise about 21 nucleotides.
24. The siNA molecule of claim 23, wherein about 19 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule and wherein at least two 3' terminal nucleotides of each fragment of the siNA molecule are not base-paired to the nucleotides of the other fragment of the siNA molecule.
25. The siNA molecule of claim 24, wherein each of the two 3' terminal nucleotides of each fragment of the siNA molecule are 2'-deoxy-pyrimidines.
26. The siNA molecule of claim 25, wherein said 2'-deoxy-pyrimidine is 2'-deoxy-thymidine.
27. The siNA molecule of claim 23, wherein all of the about 21 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule.
28. The siNA molecule of claim 23, wherein about 19 nucleotides of the antisense region are base-paired to the nucleotide sequence of the RNA encoded by a HIF1 gene or a portion thereof.
29. The siNA molecule of claim 23, wherein about 21 nucleotides of the antisense region are base-paired to the nucleotide sequence of the RNA encoded by a HIF1 gene or a portion thereof.
30. The siNA molecule of claim 9, wherein a 5'-end of the fragment comprising said antisense region optionally includes a phosphate group.

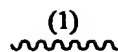
31. A composition comprising the siNA molecule of claim 1 in an pharmaceutically acceptable carrier or diluent.
32. A siNA according to claim 1 wherein the HIF1 RNA comprises Genbank Accession No. NM_001530.2.
33. A siNA according to claim 1 wherein said siNA comprises any of SEQ ID NOs. 1-562
34. A composition comprising the siNA of claim 32 together with a pharmaceutically acceptable carrier or diluent.
35. A composition comprising the siNA of claim 33 together with a pharmaceutically acceptable carrier or diluent.

1/25

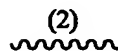
Figure 1

= SOLID SUPPORT

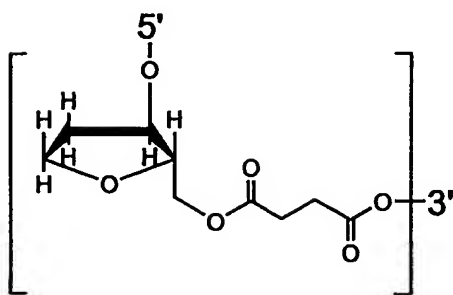
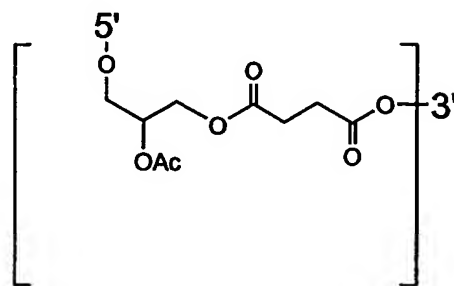
R = TERMINAL PROTECTING GROUP

FOR EXAMPLE:
DIMETHOXYTRITYL (DMT)

= CLEAVABLE LINKER

(FOR EXAMPLE: NUCLEOTIDE SUCCINATE OR
INVERTED DEOXYABASIC SUCCINATE)

= CLEAVABLE LINKER

(FOR EXAMPLE: NUCLEOTIDE SUCCINATE OR
INVERTED DEOXYABASIC SUCCINATE)INVERTED DEOXYABASIC SUCCINATE
LINKAGE

GLYCERYL SUCCINATE LINKAGE

2/25

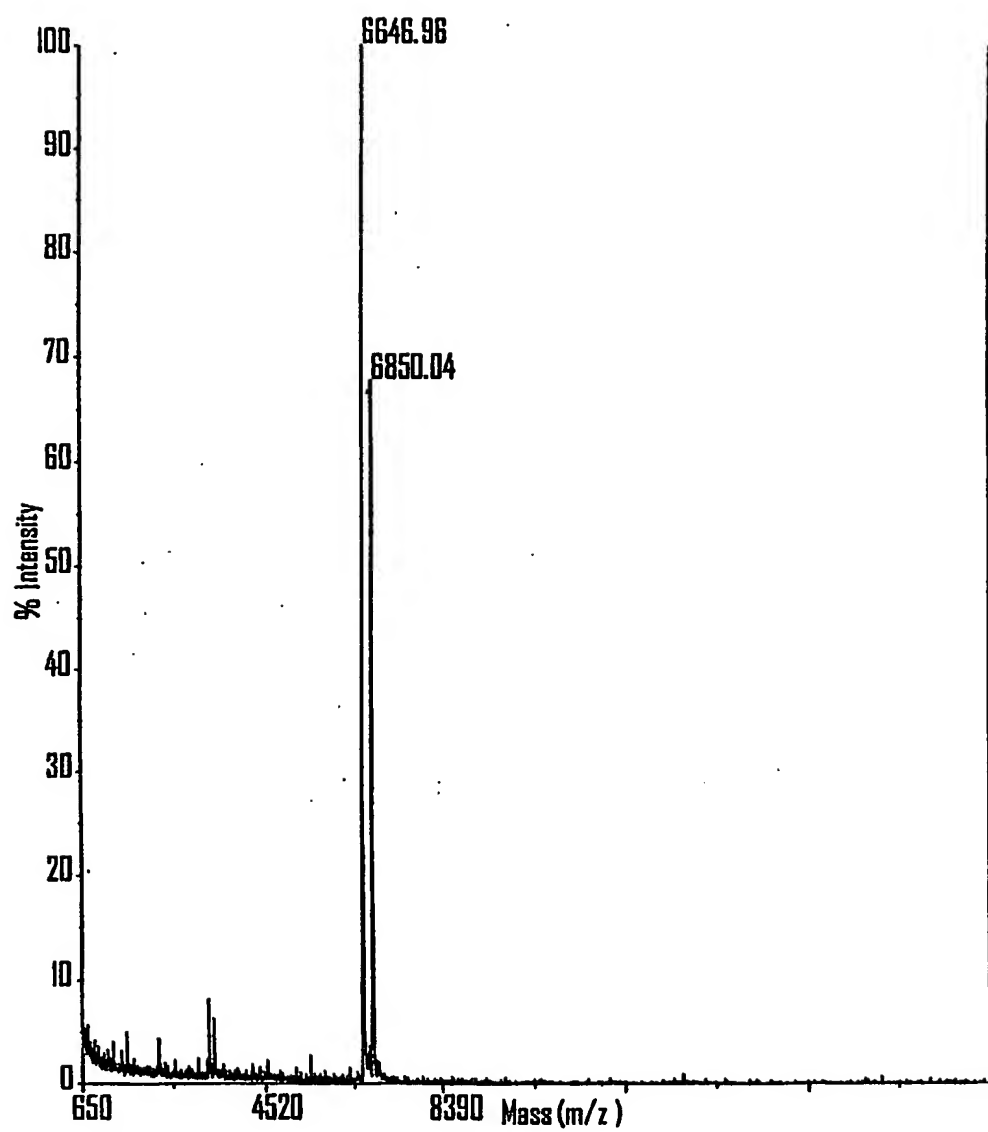
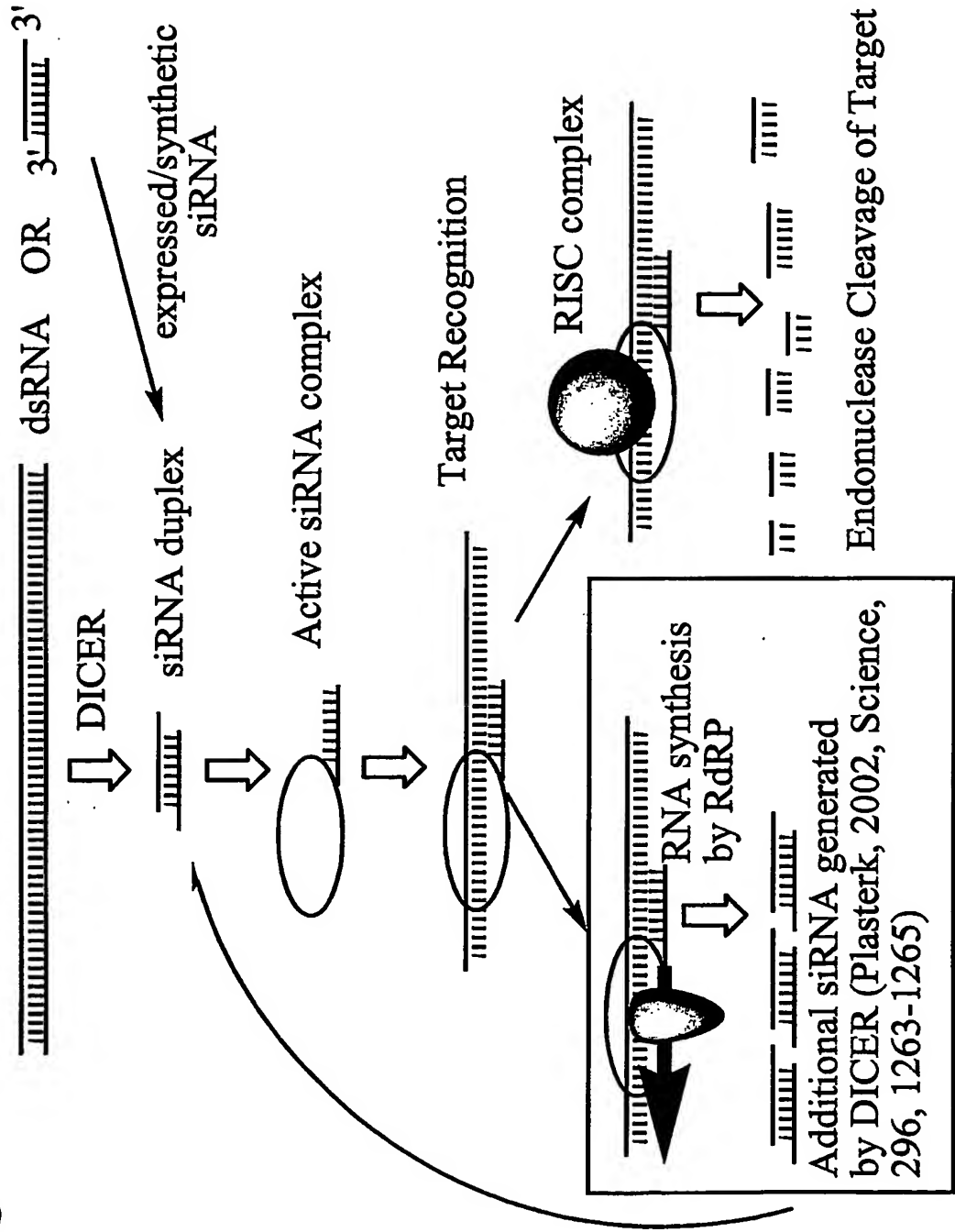
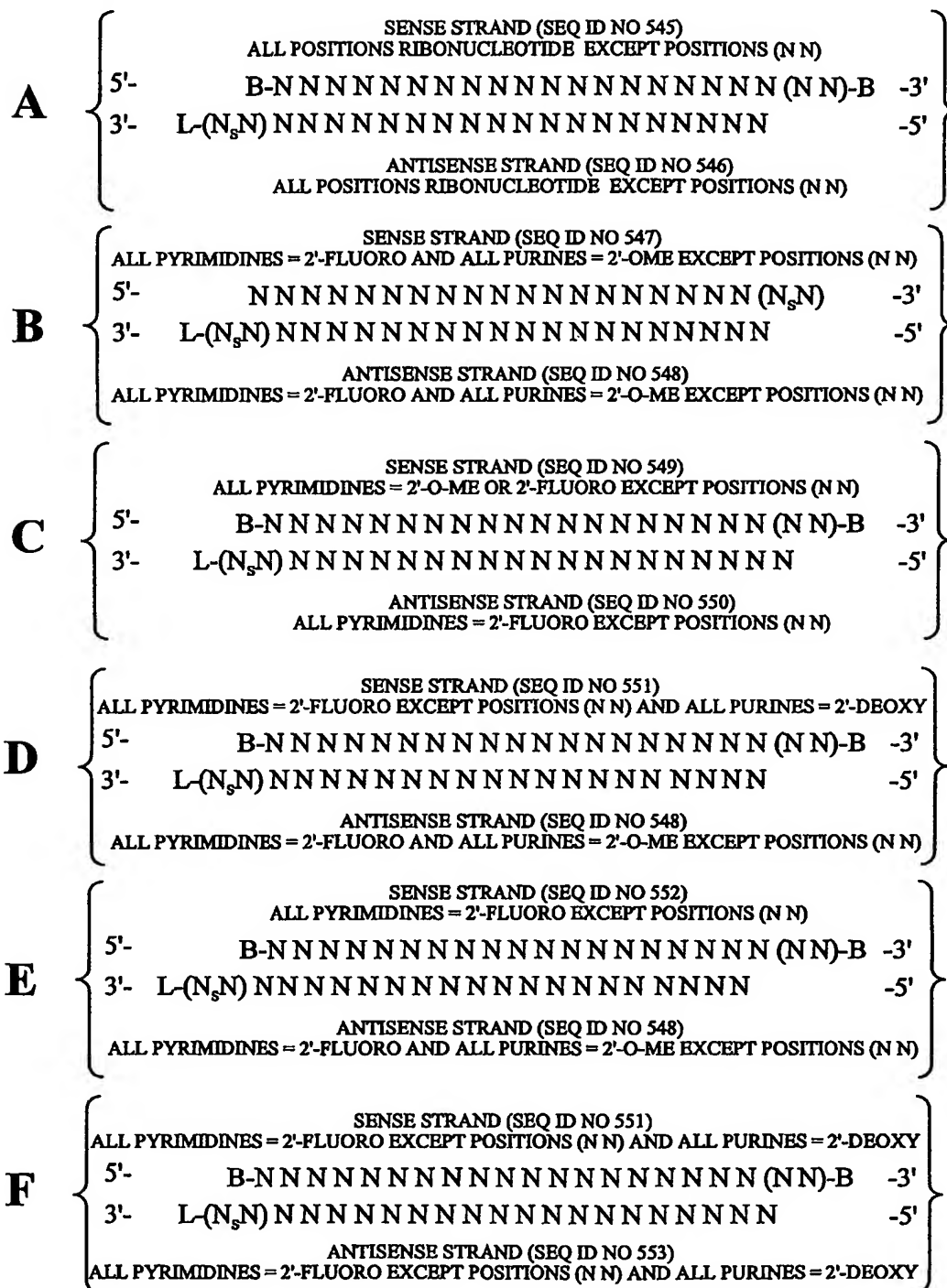
Figure 2

Figure 3



4/25

Figure 4

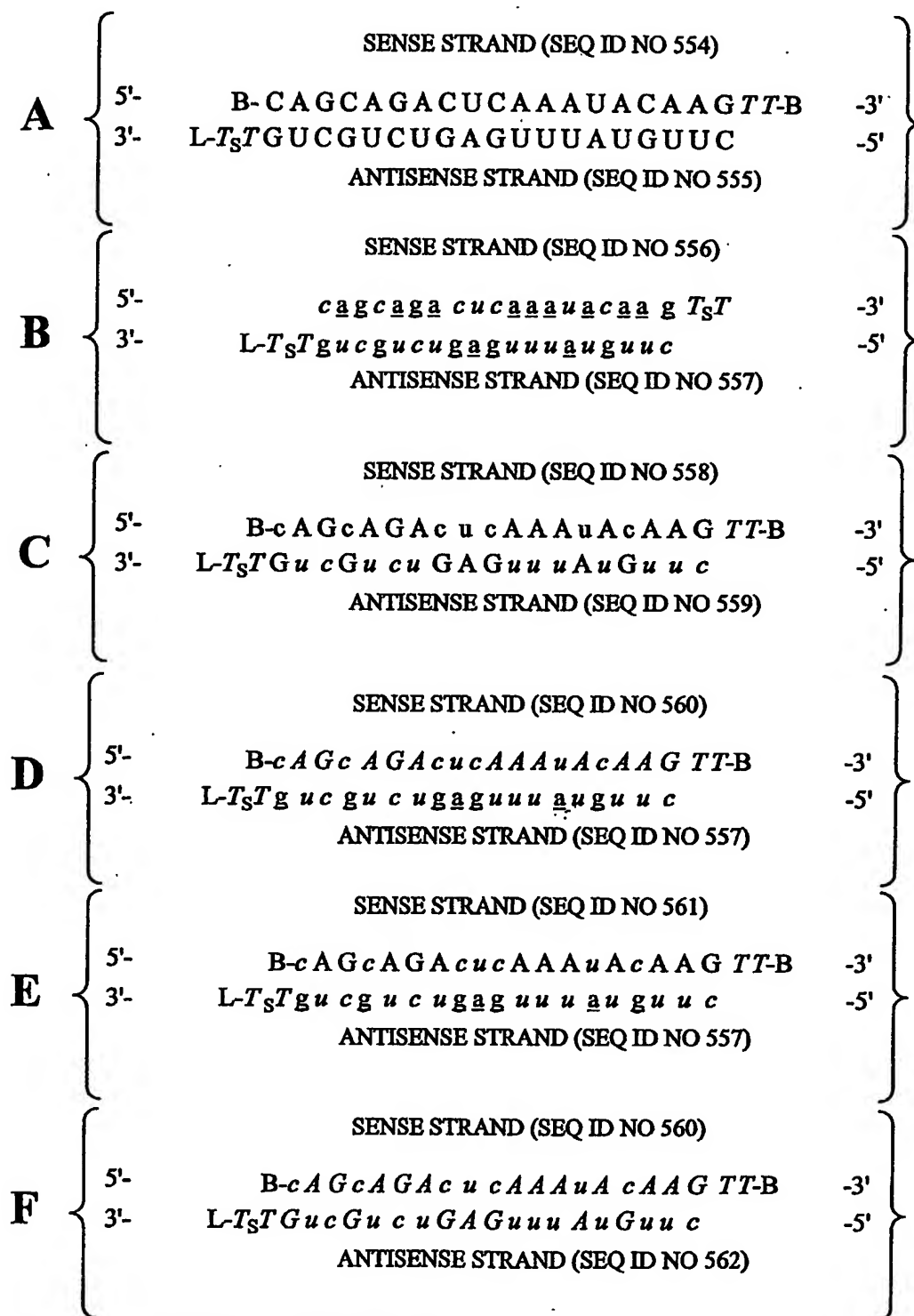
POSITIONS (NN) CAN COMPRISE ANY NUCLEOTIDE, SUCH AS DEOXYNUCLEOTIDES (eg. THYMIDINE) OR UNIVERSAL BASES

B = ABASIC, INVERTED ABASIC, INVERTED NUCLEOTIDE OR OTHER TERMINAL CAP THAT IS OPTIONALLY PRESENT

L = GLYCERYL MOIETY OR B THAT IS OPTIONALLY PRESENT

S = PHOSPHOROTHIOATE OR PHOSPHORODITHIOATE THAT IS OPTIONALLY PRESENT

5/25

Figure 5

lower case = 2'-O-Methyl or 2'-deoxy-2'-fluoro

italic lower case = 2'-deoxy-2'-fluorounderline = 2'-O-methyl**ITALIC UPPER CASE** = DEOXY**L** = GLYCERYL OR B MOIETY OPTIONALLY PRESENT**B** = ABASIC, INVERTED ABASIC, INVERTED NUCLEOTIDE OR OTHER TERMINAL CAP THAT IS OPTIONALLY PRESENT**S** = PHOSPHOROTHIOATE OR PHOSPHORODITHIOATE OPTIONALLY PRESENT

Figure 6

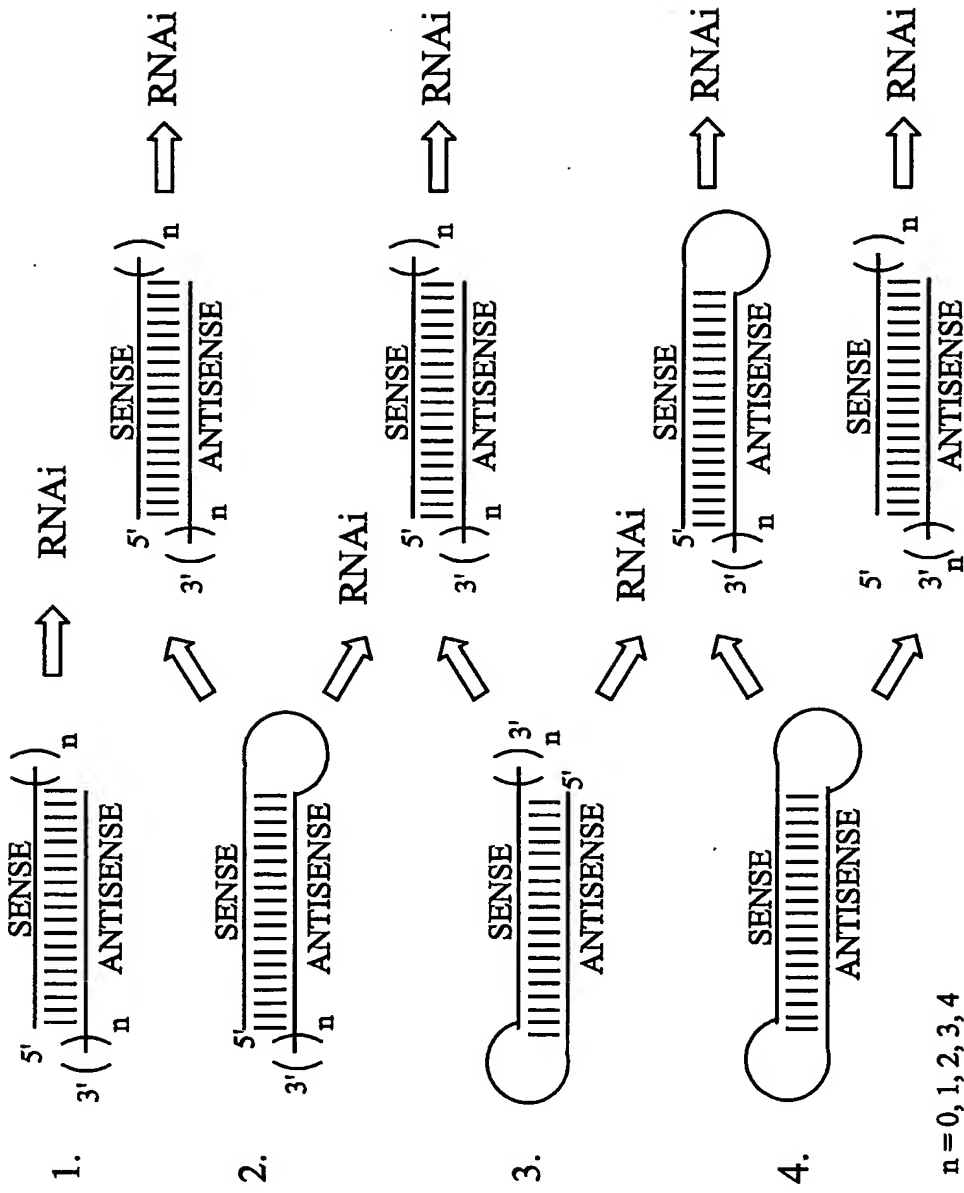
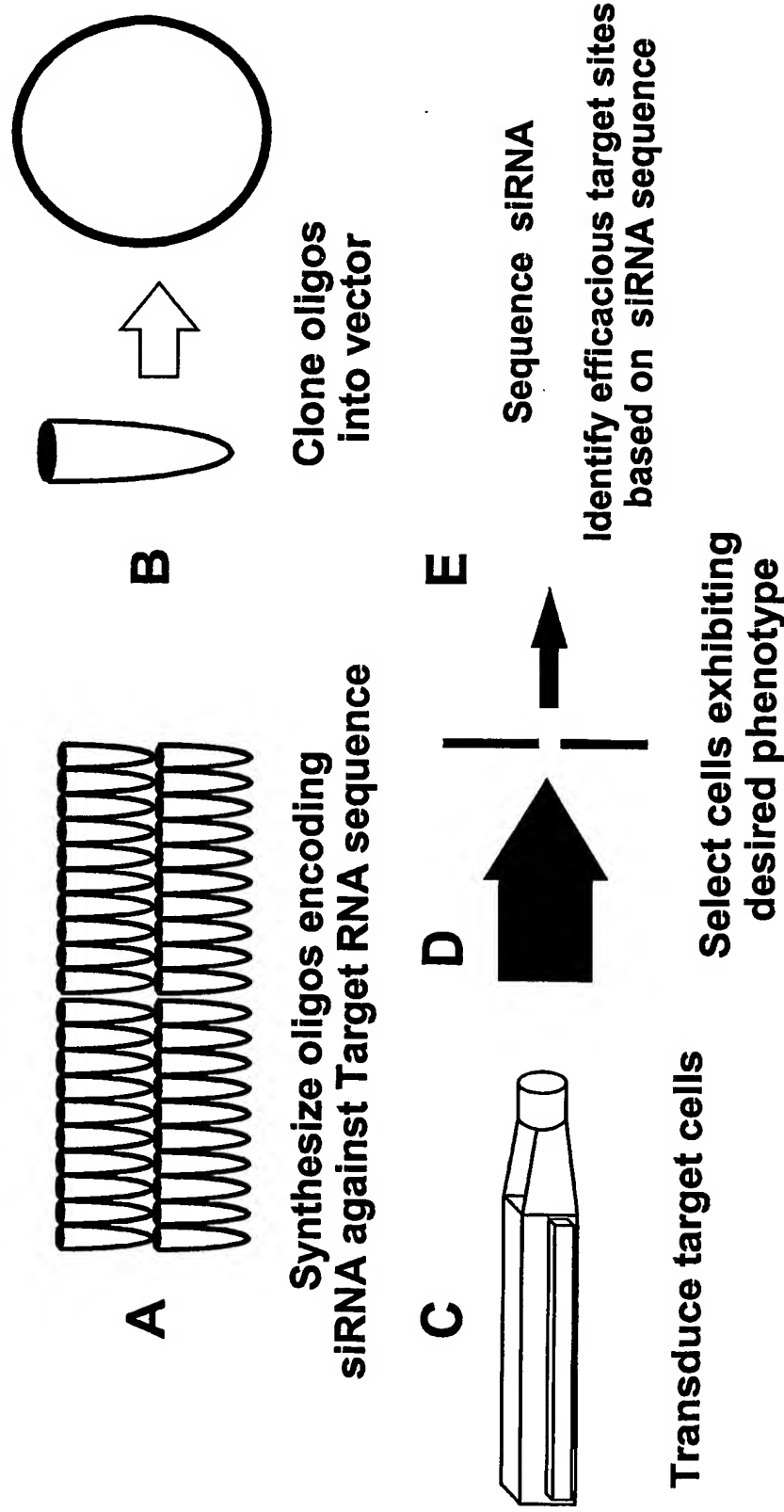
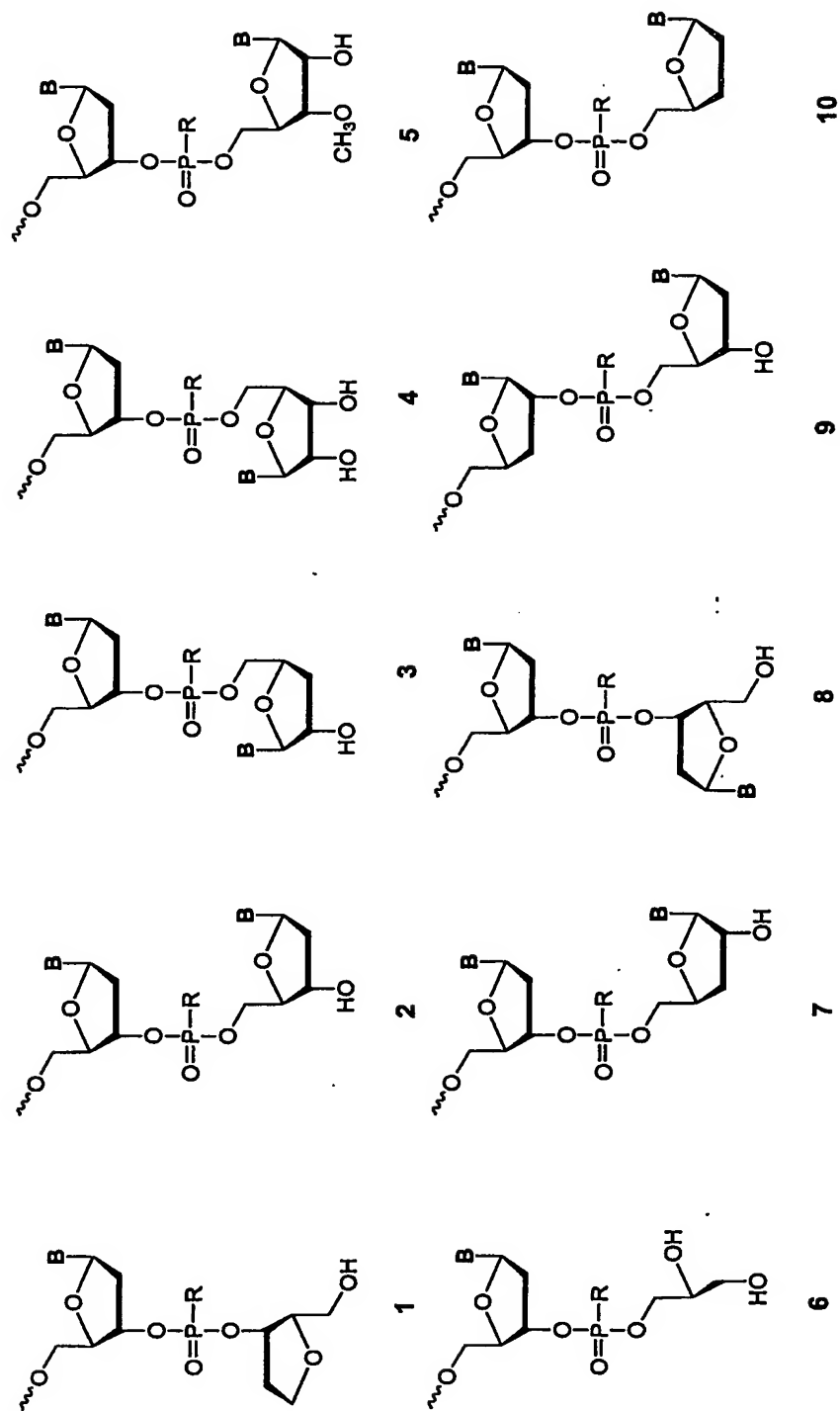


Figure 9: Target site Selection using siRNA

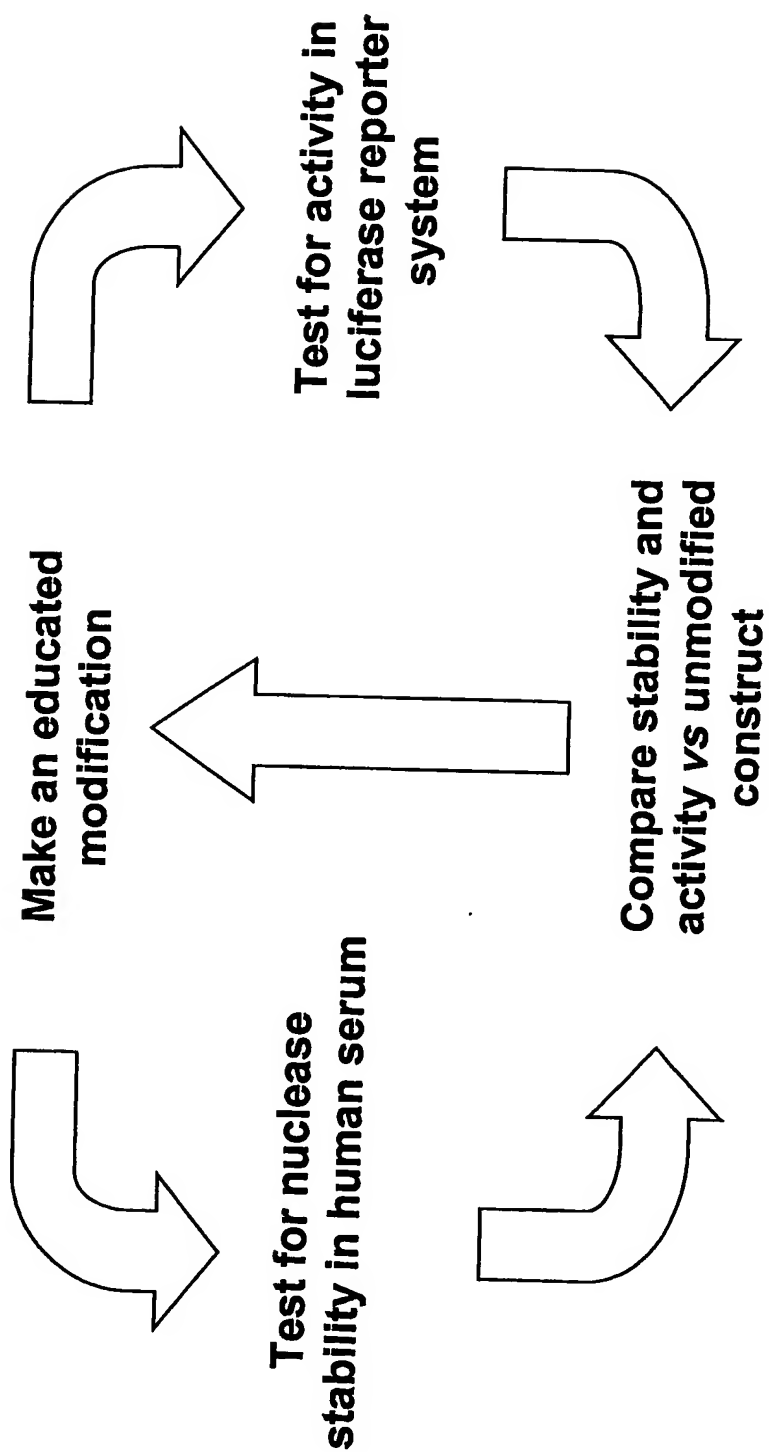


10/25

Figure 10

R = O, S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, or aralkyl
B = Independently any nucleotide base, either naturally occurring or chemically modified, or optionally H (abasic).

11/25

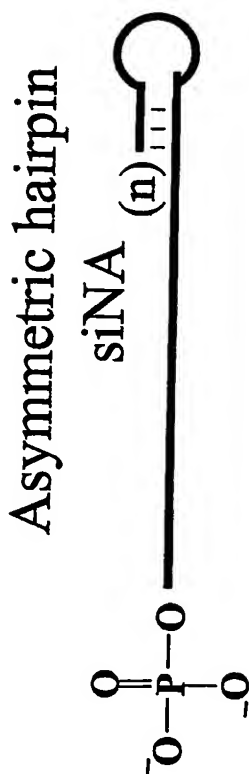
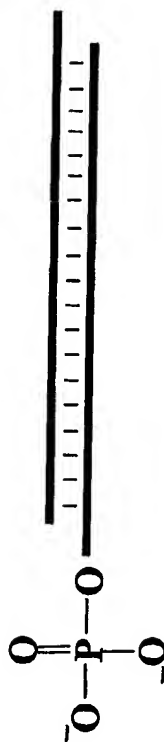
Figure 11: Modification Strategy

12/25

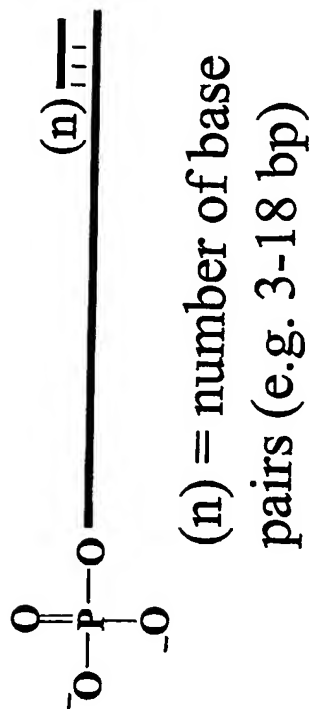
Figure 12: Phosphorylated siNA constructs



Phosphates can be modified
as described herein



Asymmetric duplex
siNA



**Figure 14A: Duplex forming oligonucleotide constructs that utilize
Palindrome or repeat sequences**

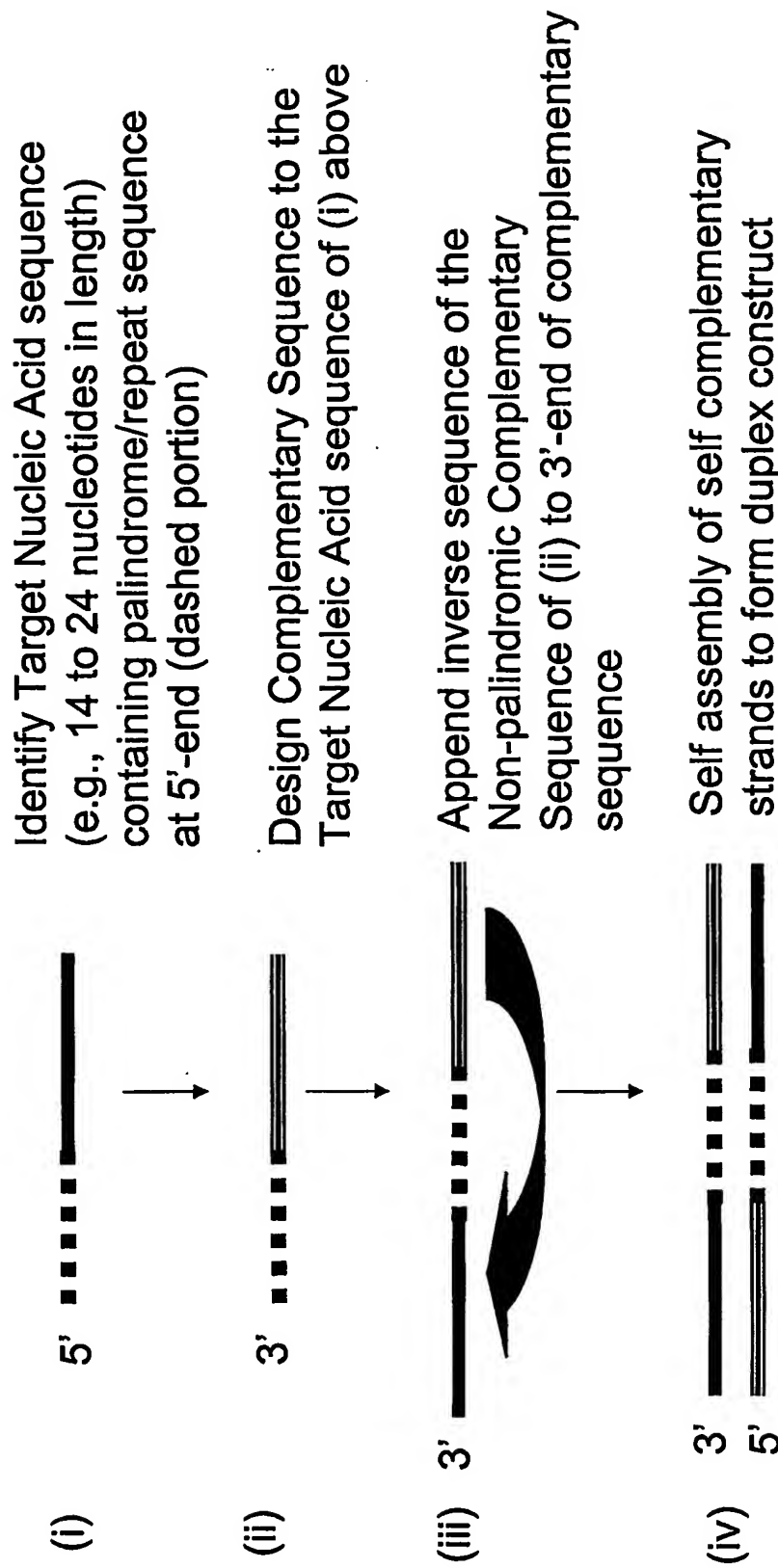


Figure 14B: Example of a duplex forming oligonucleotide sequence that utilizes a palindrome or repeat sequence

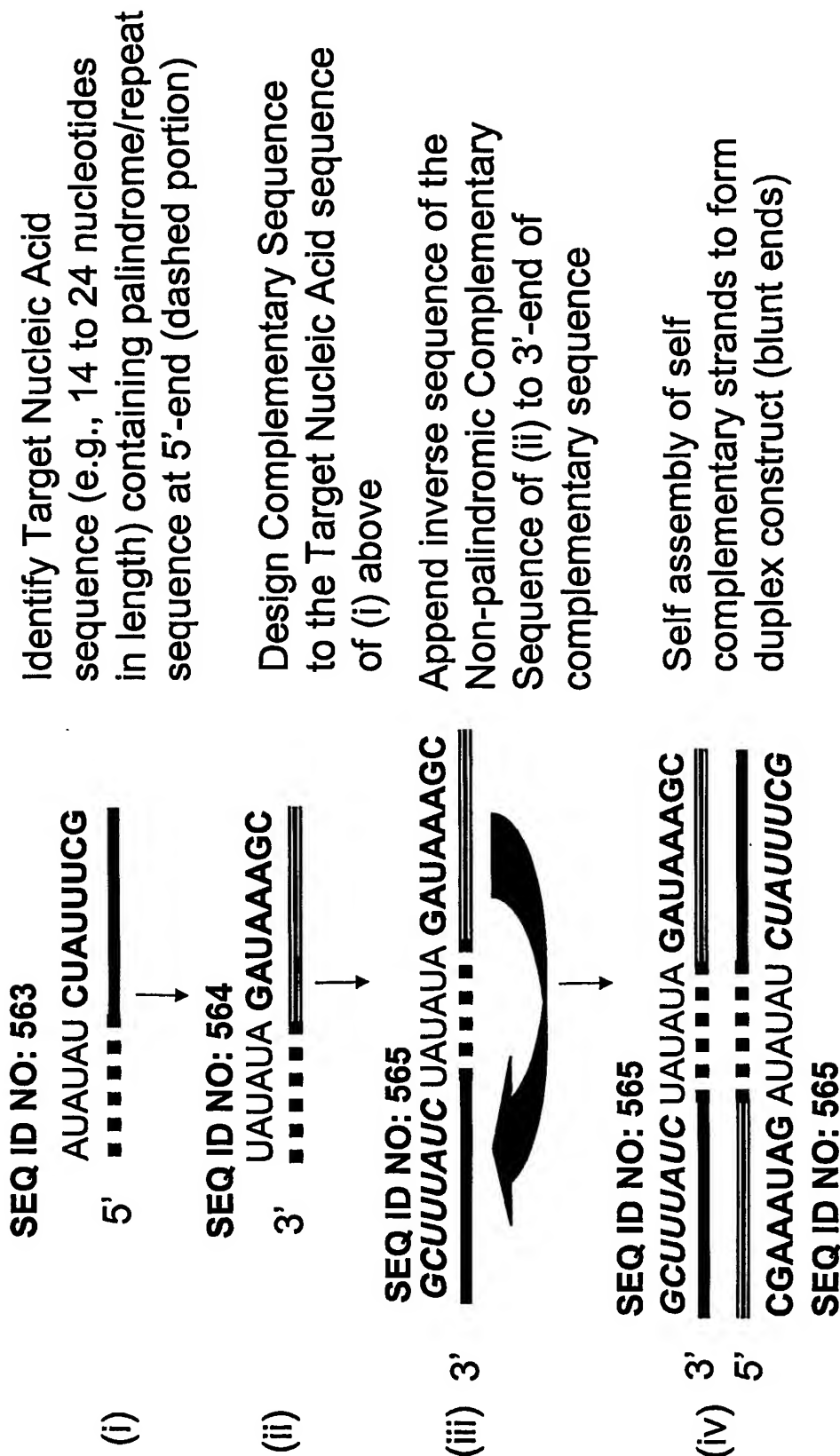


Figure 14C: Example of a duplex forming oligonucleotide sequence that utilizes a palindrome or repeat sequence, self assembly

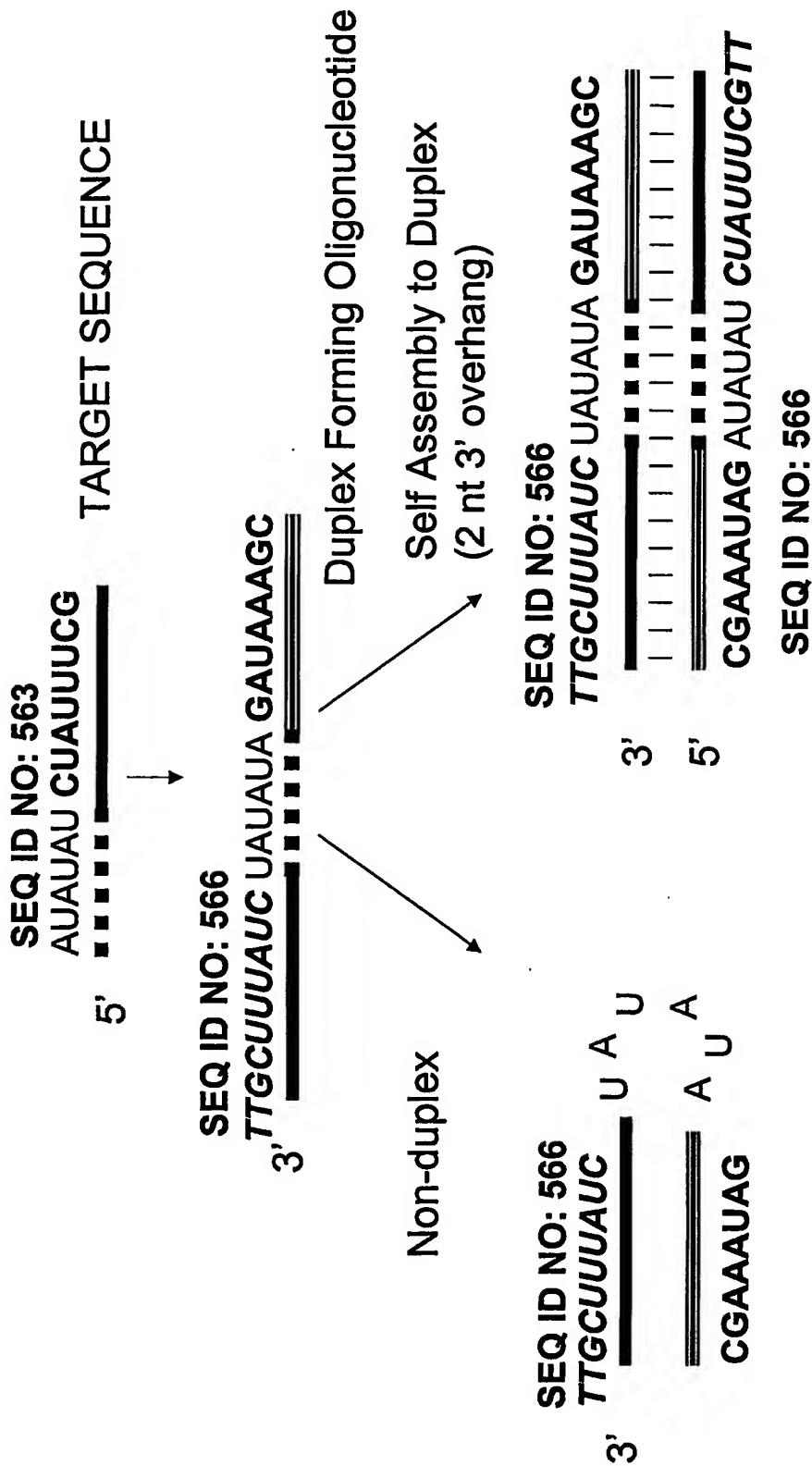


Figure 14D: Example of a duplex forming oligonucleotide sequence that utilizes a palindrome or repeat sequence, self assembly and inhibition of Target Sequence Expression



Figure 15: Duplex forming oligonucleotide constructs that utilize artificial palindrome or repeat sequences

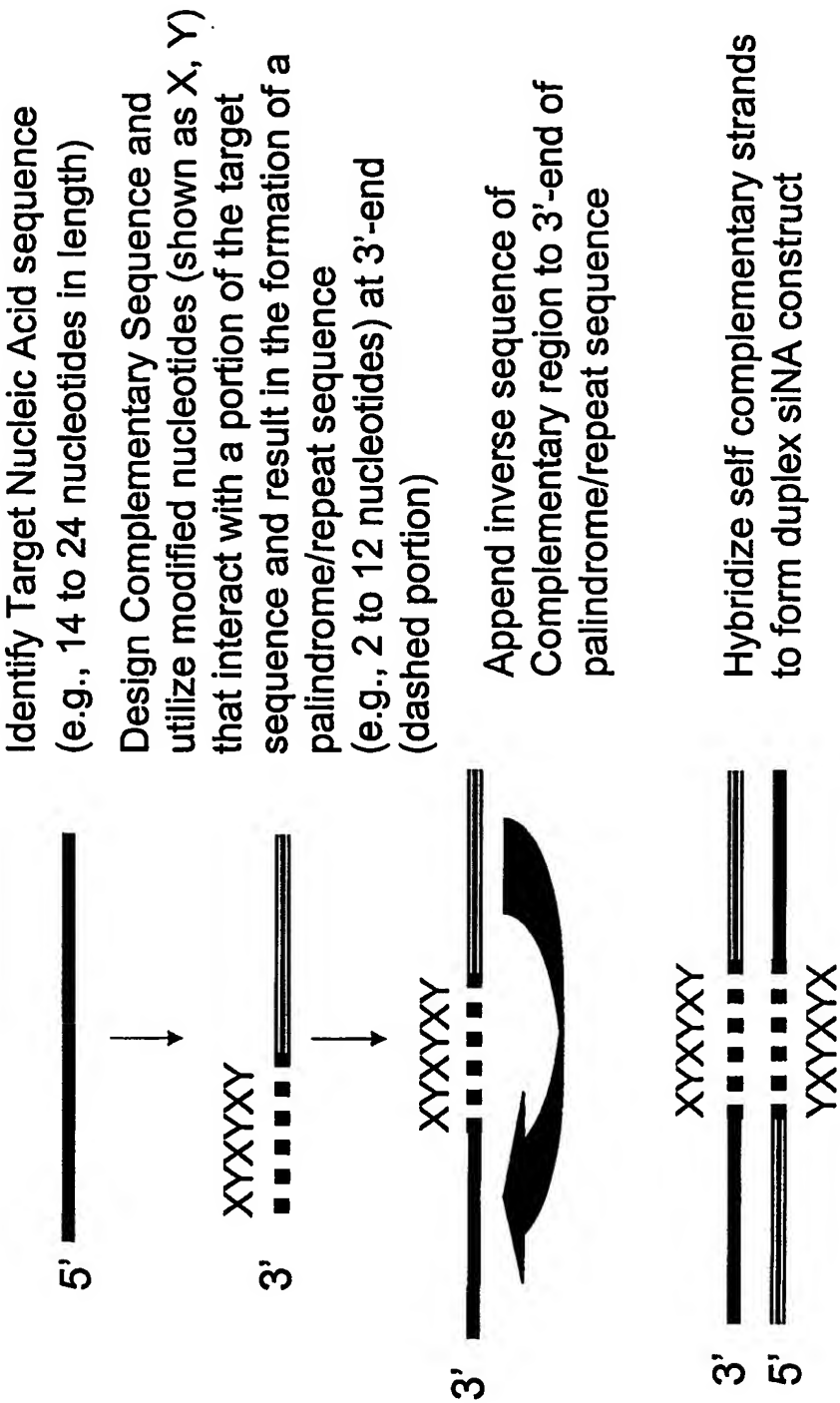


Figure 16: Examples of double stranded multifunctional siNA constructs with distinct complementary regions

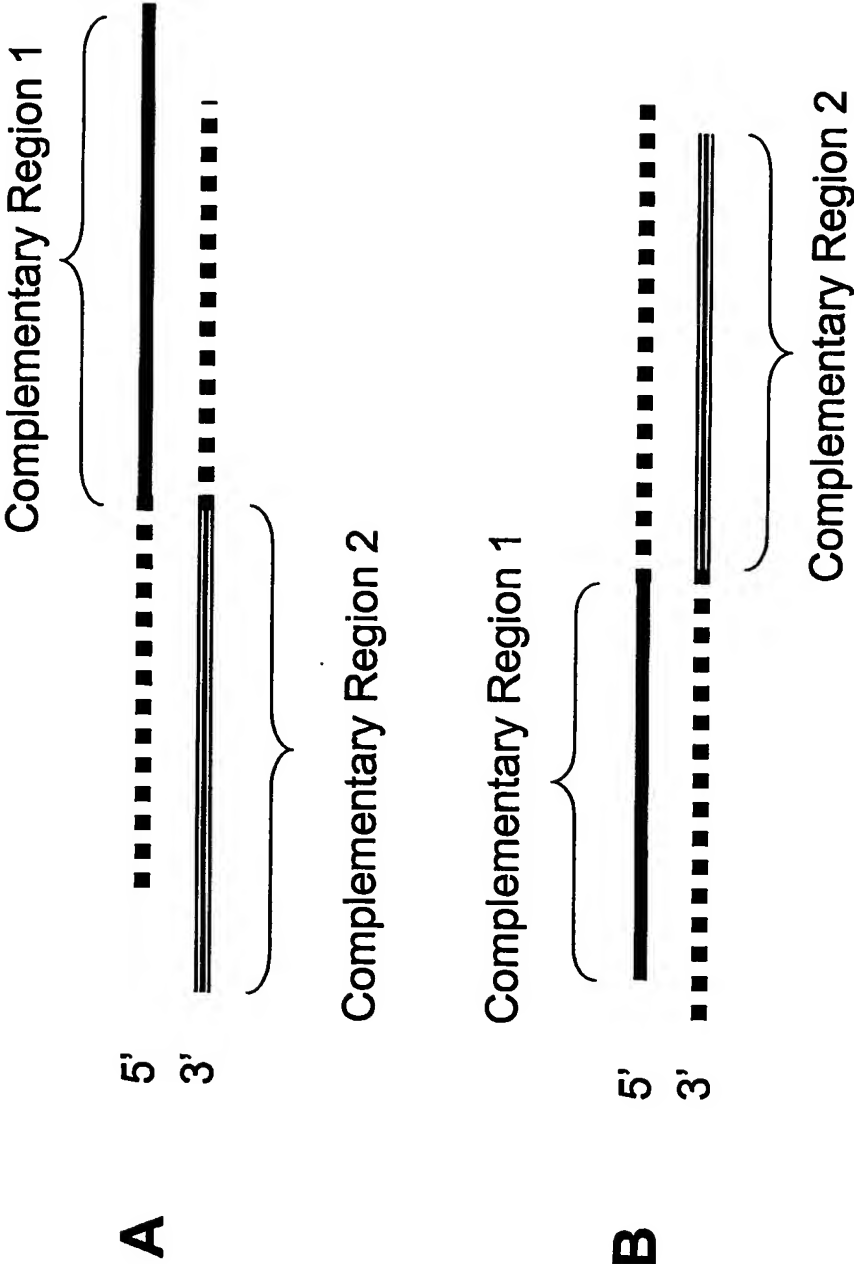


Figure 17: Examples of hairpin multifunctional siNA constructs with distinct complementary regions

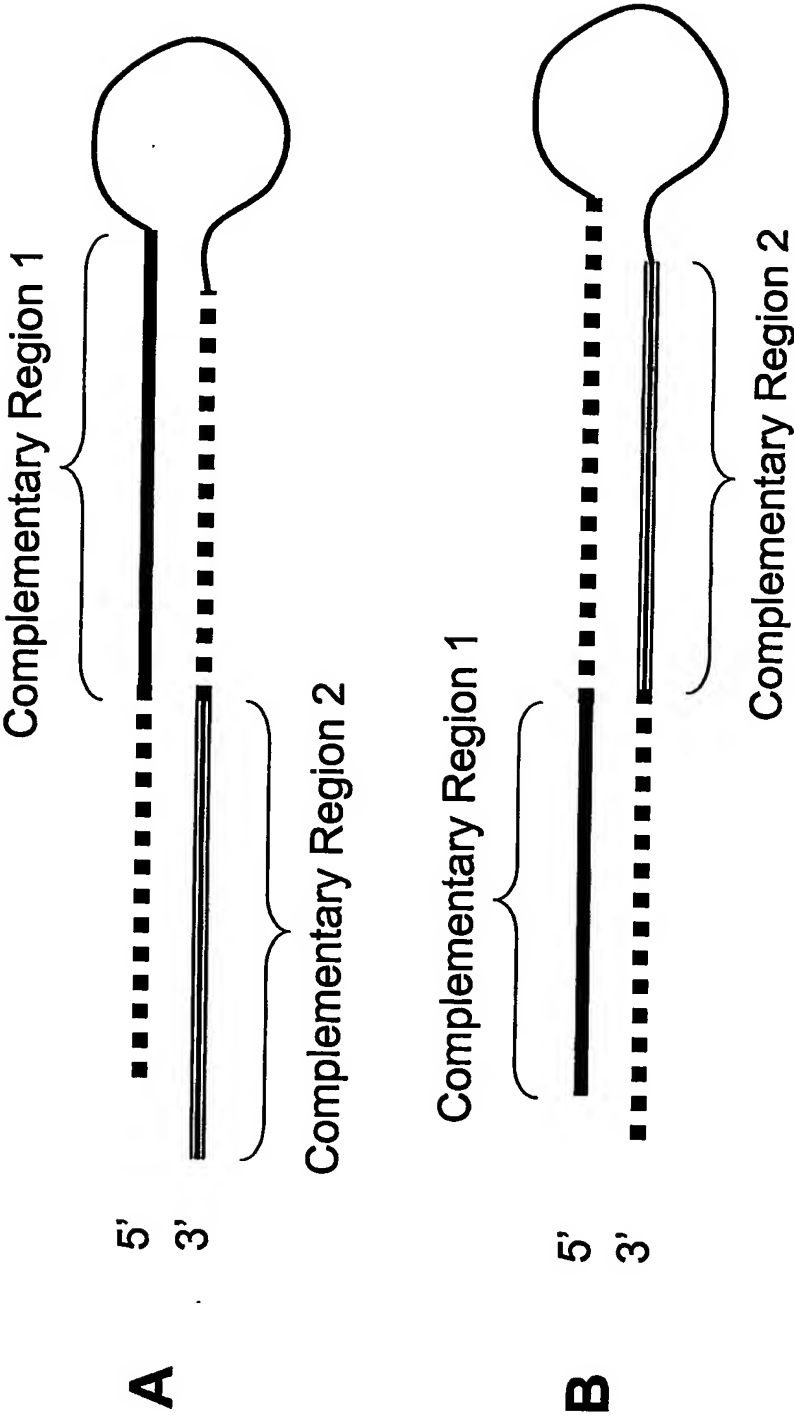


Figure 18: Examples of double stranded multifunctional siNA constructs with distinct complementary regions and a self complementary/palindrome region

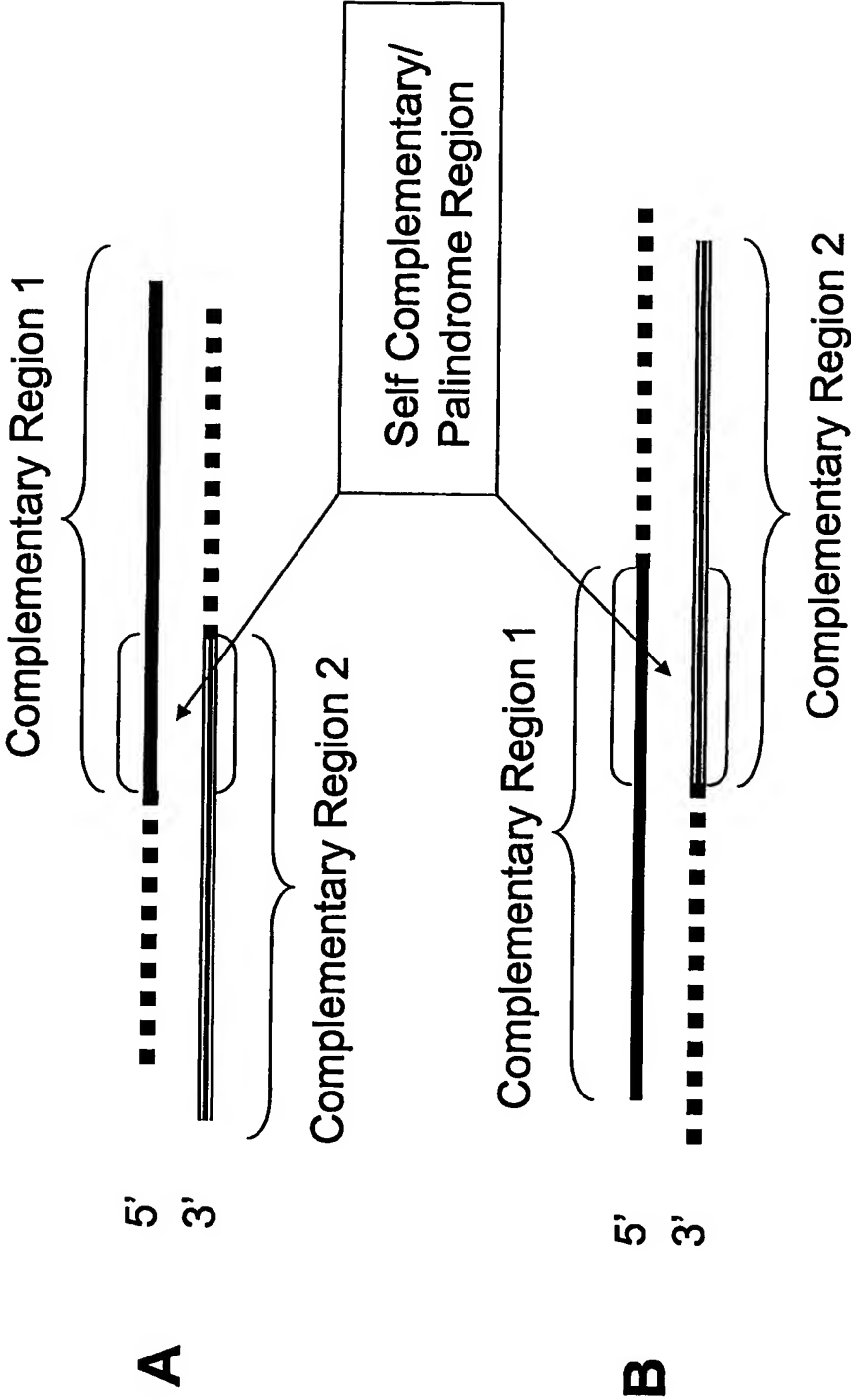
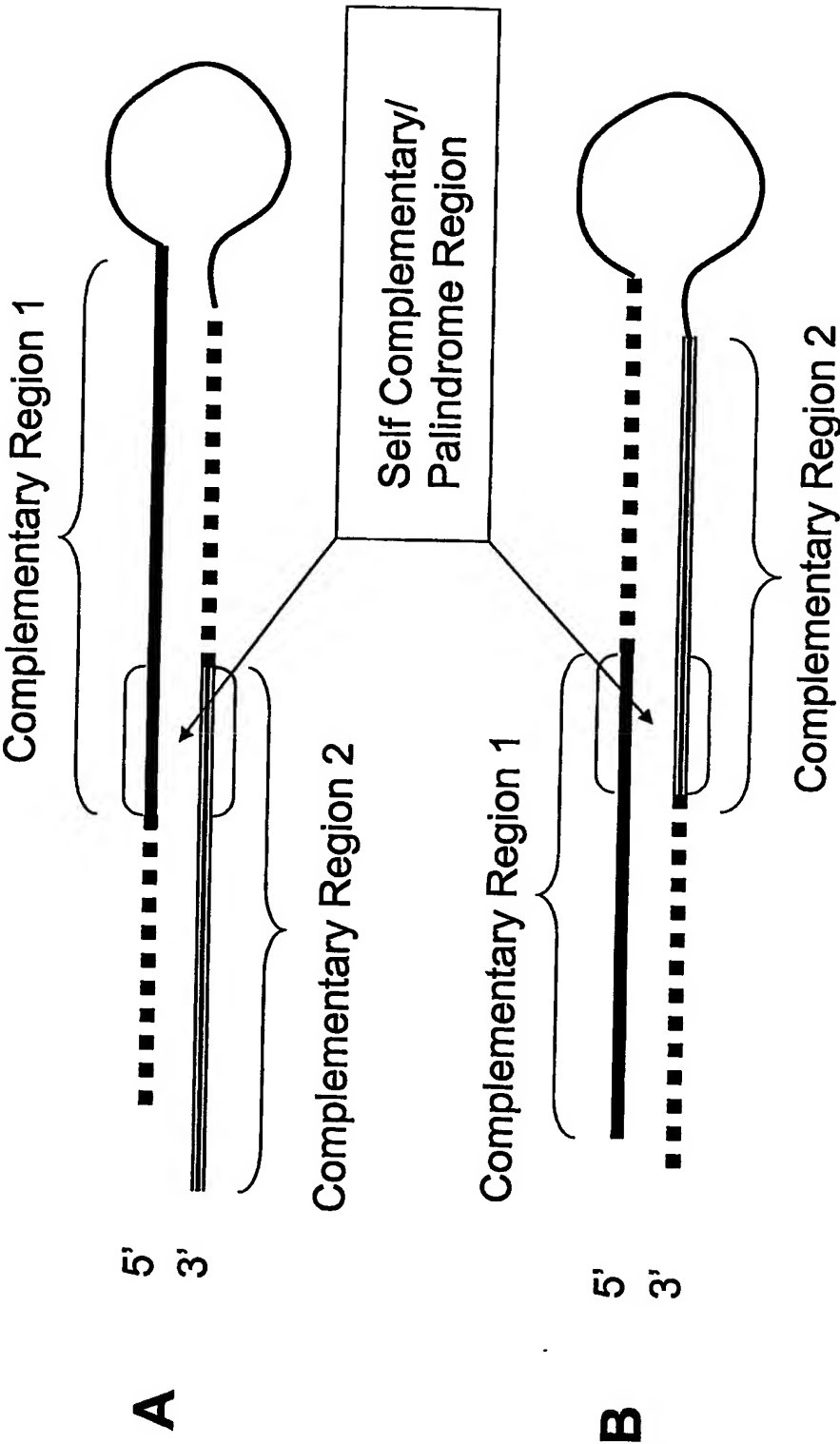


Figure 19: Examples of hairpin multifunctional siNA constructs with distinct complementary regions and a self complementary/palindrome region



**Figure 20: Example of multifunctional siRNA targeting two
Separate Target nucleic acid sequences**

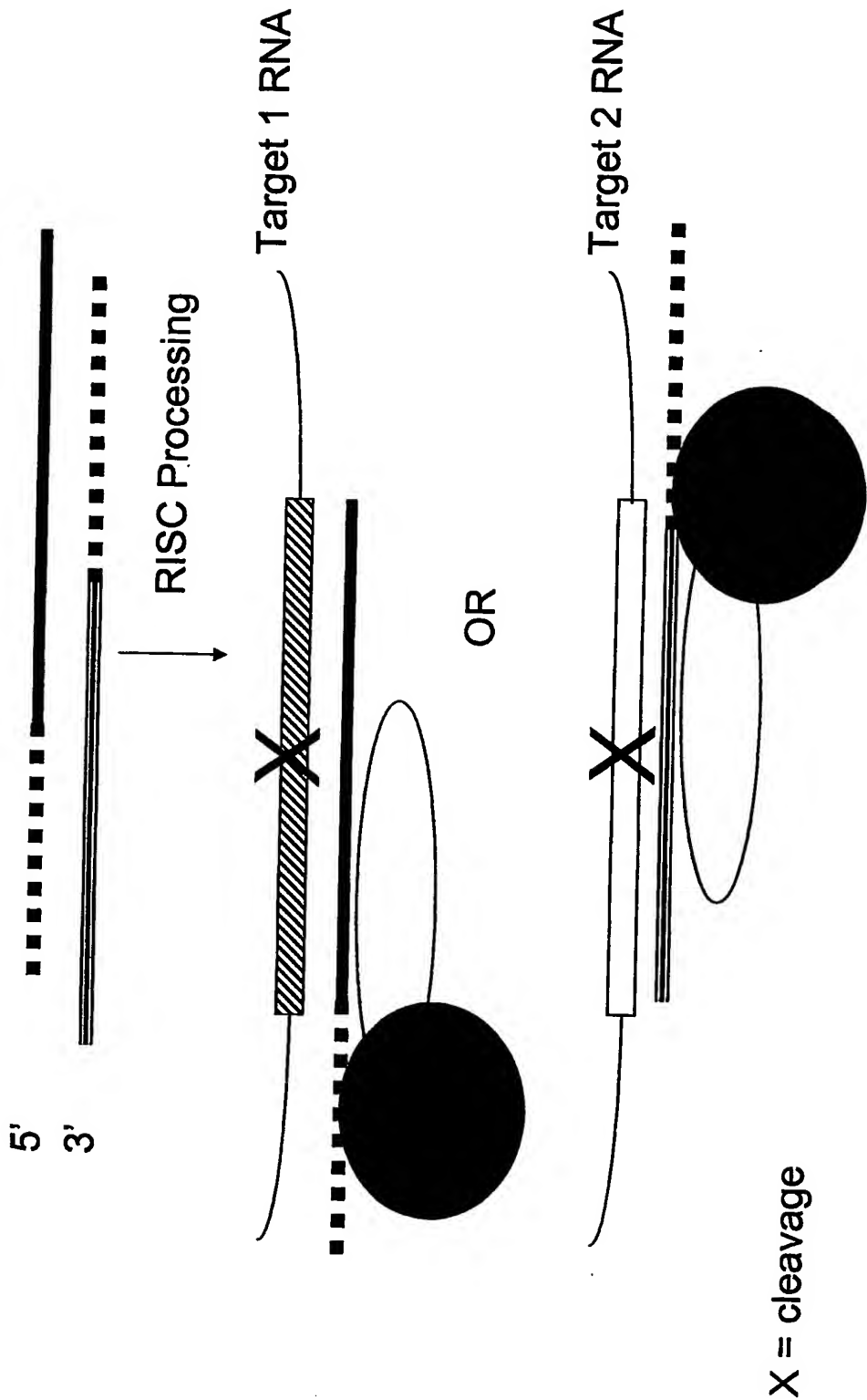


Figure 21: Example of multifunctional siNA targeting two regions within the same target nucleic acid sequence

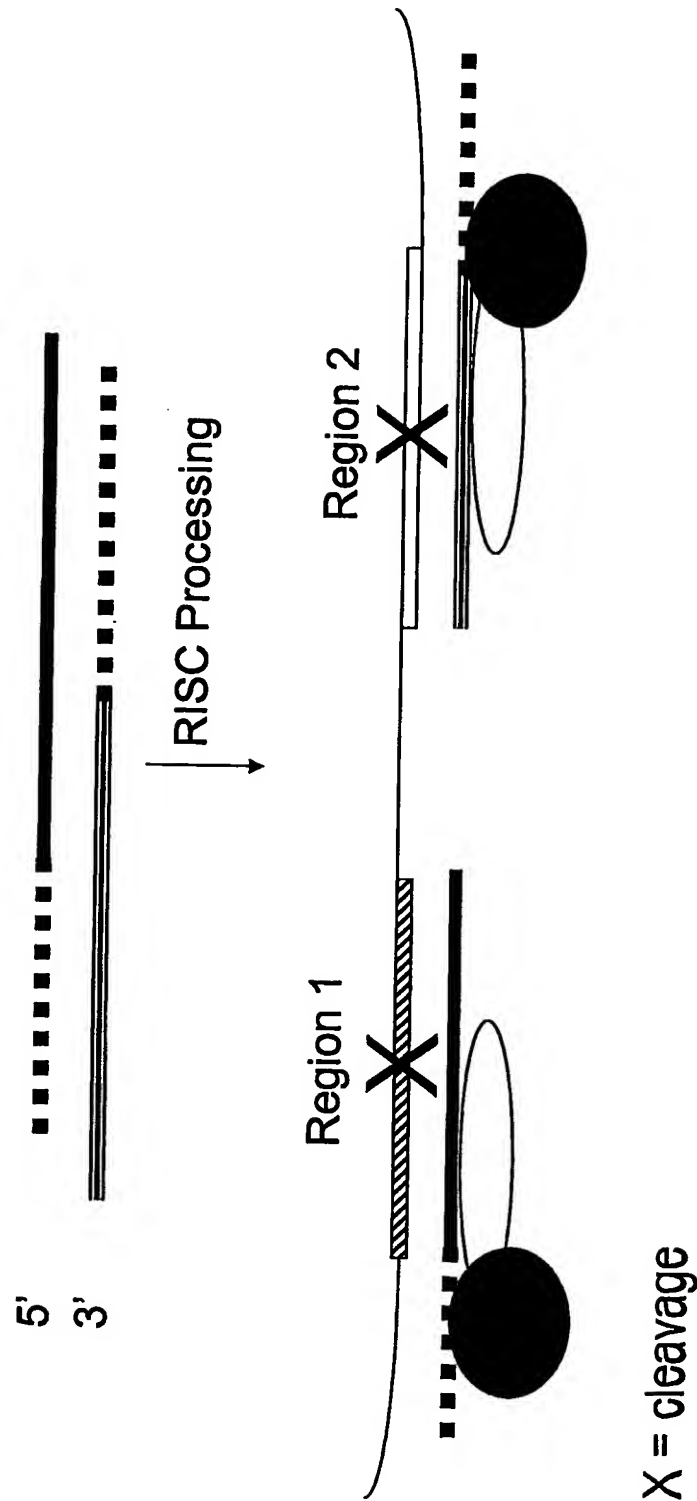


Figure 22: A549 24h HIF1 mRNA Expression
0.25 µl/well LF2K Transfection
5,000 Cells/Well

